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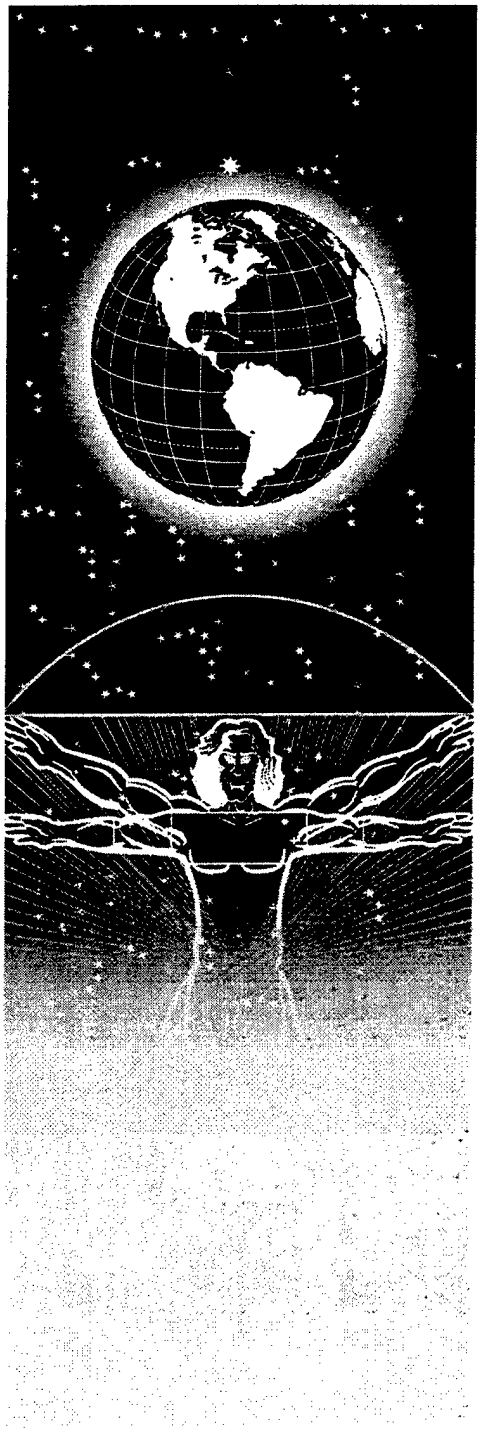

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

Genotoxicity Assays Of Ammonium Dinitramide I. *Salmonella*/Microsome Mutagenesis II. Mouse Lymphoma Cell Mutagenesis III. *In Vivo* Mouse Bone Marrow Micronuclei Test

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TECHNICAL REVIEW AND APPROVAL

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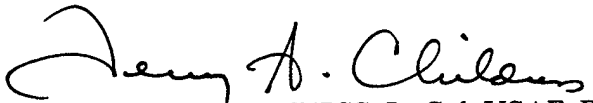
The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



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Ammonium dinitramide (ADN) was examined for its genetic toxicology effects using a battery of short-term mutagenicity screening assays, which included *Salmonella*/microsome mutagenesis (Ames test), mouse lymphoma cells mutagenesis (L5178Y-TK test), and *in vivo* mouse bone marrow micronuclei assay. Results of Ames test indicated that ADN was a base-pair substitution mutagen, causing about 2-fold (without S9) or 3-fold (with S9) increases of revertants in TA100, while there was o increase of mutants in TA1535, TA1537, and TA98. ADN also induced mutation at the TK locus of mouse lymphoma cells, causing 40-95% (without S9) or 130-220% (with S9) increases of mutants. The *in vivo* micronuclei examination revealed a dose-dependent increase of micronucleated cells in the bone marrow of both male and female mice treated with ADN in a dose range of 62.5-750 mg/kg (single dose for 3 consecutive days), with a maximal induction of 3-fold increase at the highest dose. Toxicity (determined as a decrease in PCE/NCE ratio) was observed in the same dose range. These results demonstrate that ADN is mutagenic to both bacteria and mammalian cells and causes chromosomal damage in mouse bone marrow cells *in vivo*.

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PREFACE

This document serves as a final report summarizing the work performed for the genotoxicology studies of ammonium dinitramide. The research described herein was performed by the Cellular and Molecular Toxicology Program of ManTech Environmental Technology, Inc., located in Research Triangle Park, NC, under the direction of Darol E. Dodd, Ph.D., Director of the Toxic Hazards Research Unit, located at Wright-Patterson Air Force Base, OH.

This research began in March 1994 and was completed in July 1994 under Department of the Air Force Contract No. F33615-90-C-0532 (Study No. A06). Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S.Air Force, Armstrong Laboratory, Toxicology Division. This study was cosponsored by the U.S. Army under the direction of LTC Daniel J. Caldwell, Army Medical Research Detachment, Walter Reed Army Institute of Research.

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Table of Contents:

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SUMMARY

Ammonium dinitramide (ADN) was examined for its genetic toxicology effects using a battery of short-term mutagenicity screening assays, which included Salmonella/microsome mutagenesis (Ames test), mouse lymphoma cell mutagenesis (L5178Y-TK test), and *in vivo* mouse bone marrow micronuclei assay (MN test).

Results of Ames test indicated that ADN (at a dose of 5000 ug/plate) was a base-pair substitution mutagen, causing about 2-fold (without S9) or 3-fold (with S9) increases of revertants in TA100 as compared with the controls while there was no significant increase of mutants in TA1535, TA1537 and TA98. ADN at a dose of 5000 ug/ml (highest dose tested) also significantly induced mutation at the TK locus of mouse lymphoma cells, causing 40-95% (without S9) or 130-220% (with S9) increases of trifluorothymidine (TFT)-resistant mutants. The *in vivo* micronuclei examination revealed a dose-dependent increase of micronucleated cells in the bone marrow of both male and female mice treated with ADN in a dose range of 62.5-750 mg/kg (single dose for 3 consecutive days), with a maximal induction of 3-fold increase at the highest dose. Toxicity (determined as a decrease in PCE/NCE ratio) was observed in the same dose range.

The above results demonstrate that ammonium dinitramide is mutagenic to both bacteria and mammalian cells and causes chromosomal damage in mouse bone marrow cells *in vivo*.

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I. Introduction:

The overall objective of the study is to determine the potential genotoxicity associated with the exposure to ammonium dinitramide (ADN), an explosive chemical that is being considered for potential military and space application.

Three short-term genotoxic assays are used to examine the genotoxicity of ammonium dinitramide, which include:

I-A. Salmonella/microsome mutagenesis (Ames Test)

The Salmonella/Mammalian microsome reverse mutation system is a well-defined short-term assay for the detection of carcinogens/mutagens. It measures the reversion from his- (histidine dependent) to his+ (histidine independent) induced by chemicals which cause base changes or frameshift mutations in the genome of this organism. The assay is performed in accordance with the EPA/TSCA Health Effect Testing Guidelines 40 CFR 798.5265. In this assay, bacteria are exposed to the test agent with and without a metabolic activation system (Aroclor-1254 induced rat liver S9 with co-factors) and plated onto minimum agar medium which is deficient in histidine. After incubation for 48 hours, revertant colonies are counted and compared with the number of spontaneous revertants in an untreated and/or vehicle control culture. The mutagenicity of the test agents is evident by the increased number of revertants.

I-B. Mouse lymphoma cell mutagenesis (L5178Y-TK Test)

The L5178Y mouse lymphoma-TK assay detects the mutations at the thymidine kinase locus and is used to test the mutagenicity of the test agent in mammalian cell cultures. The assay is performed in accordance with the EPA/TSCA Health Effect Testing Guidelines 40 CFR 798.5300. Thymidine monophosphate (TMP) occupies a unique position in DNA replication. Of the four principal deoxyribonucleotide monophosphates, TMP alone does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition, which serves as a regulator for DNA synthesis. If the TME is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the "salvage" enzyme thymidine kinase (TK), which normally phosphorylates thymidine to TMP in most mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues. In the mouse lymphoma cell forward mutation assay, the TK-competent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After a certain period of expression, the cells are shifted to a selective medium containing the lethal analogue trifluorothymidine (TFT). Only the mutant cells (TK-/-) can

survive under the selection condition, and the mutagenicity of the test compound is evident by the increase in the number of mutants.

I-C. *In vivo* mouse bone marrow micronucleus assay (MN Test)

The *in vivo* mammalian micronucleus test, which detects the damage of chromosome or mitotic apparatus caused by chemicals, is used to examine the chromosome-damaging effect of the test agent. Polychromatic erythrocytes (PCE) in bone marrow of rodents are used in this assay. The assay is based on an increase in the frequency of micronucleated PCEs in bone marrow of the treated animals. The assay is conducted according to the EPA/TSCA Health Effect Testing Guidelines 40 CFR 798.5395. Micronuclei are small particles consisting of acentric fragments of chromosome or entire chromosomes, which lag behind at anaphase of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. The clastogenic effect or mitotic apparatus damaging effect of the test agent will be evident by the increased frequency of micronucleated PCEs in the bone marrow.

Section II. METHODS

The methodology for the three assays was given in details in the **" Protocols for the Genotoxicity Assays of Ammonium Dinitramide (ADN)"** (Appendix D), and briefly described as follows.

II-A. Salmonella/microsome mutagenesis (Ames Test)

a. Genotype confirmation

Genotypes of each strain were confirmed prior to the mutagenesis study, which included the requirement of histidine (His-), the sensitivity to crystal violet (rfa mutation) and U.V. light (uvrB mutation), the resistance to ampicillin (R factor), and the occurrence of spontaneous revertants.

b. Mutagenicity assay - Plate incorporation

A preliminary range-finding assay was performed using TA100 to determine the test doses of ADN. Four tester strains were used in the mutagenicity assay, which include TA1535, TA100, TA1537 and TA98. Ammonium dinitramide dissolved in distilled water was tested in all 4 tester strains at a dose range of 0.3125-5 mg/plate with and without S9 activation. The bacterium was cultured in nutrient broth at 37°C water bath with shaking for 10-12 hours. 0.1 ml of the culture was added to 2 ml of top agar which was melt and held at 45°C heating block, along with 0.1 ml of the test agent, and 0.5 ml of S9 mixture (in S9+ plates only). The contents were mixed and then poured onto the surface of a minimum glucose agar plate and spreaded out evenly. The top agar was allowed to solidify and the plates were incubated at 37°C for 48 hours before the number of revertants per dish was counted by an automatic colony counter. Cultures were set up in triplicates, and a second independent experiment was also conducted. Appropriate positive controls were included in each test (2-Aminofluorene with S9, 20 ug/plate for both TA100 and TA98; sodium azide without S9, 2 ug/plate for TA1535; 9-Aminoacridine without S9, 10 ug/plate for TA1537).

II-B. Mouse lymphoma cell mutagenesis (L5178Y-TK Test)

a. Cell culture and maintenance

The L5178Y $TK^{+/-}$ mouse lymphoma cells were maintained as suspension culture in F_{10p} media in culture flasks equilibrated with 5% CO_2, 95% air and incubated at 37°C in a rotary shaker. Each week the cells were grown in the F_{10p} media containing THMG (thymidine, hypoxanthine, methotrexate and glycine) to select against newly arising TK-/- mutants, and then placed in the F_{10p} media containing THG (thymidine, hypoxanthine, and glycine) for 1-3 days prior to use in mutagenesis study.

b. Mutagenicity assay

The doses of ADN in the mutagenesis study were determined by a preliminary range finding assay using cell growth as the indicator for toxicity. In the mutagenesis studies, cells ($6x10^6$ cells in 10 ml medium for each culture) were treated with test agents with and without S9 mixture as the activation system, and incubated at 37°C with rotation for 4 hours. After the removal of test agents and washing, cells were maintained in non-selective medium at a density of $3x10^5$ cells/ml in roller drum for 2 days at 37°C, with cell density checked daily and adjusted to $3x10^5$ cells/ml. Cells were then seeded onto soft agar medium to determine the survival and the mutation frequency. For each dose group, 3 cultures containing 200 cells/dish in non-selective medium were set up for viability measurement, another set of 3 cultures with $1x10^6$ cells/dish in selective medium containing TFT were used for mutant counting. Dishes were incubated at 37°C in an atmosphere of 5% CO_2, 95% air for 11 days before the counting and sizing of the mutants using an automatic colony counter. The mutant frequency was calculated and adjusted based on the survival percentage. Ethyl methanesulfonate (EMS, 250 nl/ml) without S9, and 3-methylcholanthrene (3-MCA, 2.5 ug/ml) with S9, were used as the positive controls in the assays. A second experiment was repeated separately.

II-C. *In vivo* mouse bone marrow micronucleus assay (MN Test)

Swiss CD-1 mice (5 males and 5 females per group), 8-10 weeks old, were used in the study. Ammonium dinitramide dissolved in distilled water was administered by gavage dosing for 3 consecutive days (62.5-750 mg/kg/dose). Cyclophosphamide (20 mg/kg/dose by i.p. injection) was used as the positive control. Twenty-four hours after the last dosing, mice were sacrificed, bone marrow cells were collected from the femur and smears were prepared by the air-dry methods. The slides were stained by May-Gruenwald/Giemsa solution and coded. The frequency of micronucleated cells were observed in 1000 polychromatic erythrocytes (PCE) per animal. The PCEs/NCEs ratio was determined by counting 1000 erythrocytes, and used as the indicator of toxicity. Micronuclei are defined as round bodies in cytoplasm with a diameter of 1/20 to 1/5 of an erythrocyte. They stain intensively, similar to the staining of the main nuclei in the nucleated cells.

Section III. Good Laboratory Practice and Quality Assurance

All assays were conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA/TSCA) Good Laboratory Practice (GLP) Standards as defined in the Federal Register (40 CFR, Part 792, 1992) and the TSCA Test Guidelines (40 CFR 798.5265, 40 CFR 798.5300, and 40 CFR 5395, 1992). All the procedures were performed in accordance with the Standard Operating Procedures (SOPs) of ManTech Environmental for the Salmonella/microsome mutagenesis assay, L5178Y mouse lymphoma cell mutagenesis and mouse bone marrow cell micronucleus test.

The Quality Assurance Auditor Ms. Sue Carl documented inspections on all procedures used in this study.

Date of Inspection	Items/Activities Inspected
A. Salmonella/microsome mutagenesis assay:	
May 24, 1994	Culture of tester strains Preparation of frozen stocks
May 25, 1994	Confirmation of genotypes Range finding assay for ADN
June 8, 1994	Mutagenesis assay (plate incorporation)
B. Mouse lymphoma cell mutagenesis assay:	
May 17-18, 1994	Range finding assay for ADN
June 7-9, 1994	Mouse lymphoma cell mutagenesis
C. In vivo mouse bone marrow micronuclei test:	
April 4-6, 1994	Chemical exposure
April 7, 1994	Collection of bone marrow Preparation of smears
April 8, 1994	Slide staining
April 8, 1994	Scoring of micronuclei and PCE/NCE

Susan L Carl
Sue Carl
Quality Assurance Auditor

7/12/94
Date

Section IV. RESULTS

IV-A. Salmonella/microsome Mutagenesis (Ames Test)

The raw data for the Ames test are attached as Appendix A, and the salient results are summarized as follows.

1. Genotype Identification:

Different genotypes of the tester strains were verified by the standard procedure of B.N. Ames prior to the study. Results confirmed that all the tester strains are qualified for the study.

Genotypes	TA98	TA100	TA1535	TA1537
Histidine requirement	+	+	+	+
rfa mutation	+	+	+	+
uvrB mutation	+	+	+	+
R factor	+	+	-	-
Spontaneous revertants	36±3	142±14	15±2	8±1

2. Dose selection for ADN:

Five log doses of ADN were tested in TA100 for dose selection, and the results are listed as follows. The top dose (5 mg/plate) did not show toxicity to the tester strain, and was selected as the top dose in the formal study along with five 2-fold dilutions.

ADN (mg/plate)	Revertants (mean ± SD of triplicates)
0	142 ± 14
0.0005	148 ± 5
0.005	155 ± 31
0.05	152 ± 10
0.5	160 ± 9
5	333 ± 30

3. Mutagenicity Assay of ADN:

The results of TA100, TA98, TA1535 and TA1537 are summarized in Tables 1 through 4, where the data are expressed as the average revertant number per plate from the triplicates.

Ammonium dinitramide significantly increased the revertant number in TA100 (with and without S9) in a dose-dependent manner, as shown in Fig.1 and Fig.2. The maximal induction by 5 mg/plate of ADN was approximately 2-fold without S9, and further increased to 3-fold with the addition of S9. The compound was negative in the other 3 tester strains.

4. Conclusion:

The above results indicate that ammonium dinitramide was mutagenic to TA100, causing base-pair substitution mutation in Salmonella.

IV-B. Mouse Lymphoma cell Mutagenesis (L5178Y-TK Test)

The raw data of the L5178Y-TK test are attached as Appendix B, and the important findings are listed as follows.

1. Result of Range Finding for ADN

The results of the range finding assay for ADN are listed in Table 5. Based on the data on cell growth, the highest dose tested (5000 ug/ml) showed about 30% inhibition in cell growth ($p<0.001$), and thus was selected as the top dose in the mutagenesis study, which included 5 two-fold diluted concentrations.

2. Results of Mutagenesis Assays for ADN

Table 6 summarizes the results of the two mutagenesis assays for ADN. ADN at doses above 250 ug/ml significantly increased the mutant number in one or both experiments. The mutation induction was not dose-dependent. Without S9 activation, ADN at 5000 ug/ml increased the mutation frequency at TK locus by approximately 40-95%. The mutagenicity of ADN was further enhanced by the addition of S9 activation system, as indicated by a 130-220% increase of the TFT resistant mutant frequency over the controls.

The size-distribution of the TFT-resistant mutants are shown in Figures 3 through 6. It is evident from Fig.3 and Fig.4 that the two positive reference mutagens, ethyl methanesulfonate (EMS, 250 nl/ml without S9) and 3-methylcholanthrene (3-MCA, 2.5 ug/ml with

S9) mainly showed a single peak of larger mutant size (0.5 mm), while ammonium dinitramide (with and without S9, see Fig. 5 and Fig. 6) showed both a peak for smaller size mutants (0.1-0.2 mm) and a peak for larger size mutants (0.5-0.6 mm).

3. Conclusion & Discussion

Results indicate that ammonium dinitramide significantly increases the TFT-resistant mutant frequency in mouse lymphoma cells. The mutagenicity of ADN is further enhanced by the S9 activation. Two peaks of mutant size (small and large) are observed in ADN-induced mutants. It has been suggested that small mutants may arise from the induction of chromosomal damage, while the large mutants arise from gene mutation (Clive et al., 1979, Mutation Res., 59, 61-108). The fact that ADN is able to induce gene mutation in Salmonella as well as chromosomal damage detected by the micronuclei assay in mouse supports the above speculation about size distribution and the mechanism of action.

IV-C. *In Vivo* Mouse Bone Marrow Micronuclei Test (MN Test)

The raw data of the MN test are attached as Appendix C, and the results are summarized as follows.

The dose of ADN in the micronuclei test was selected based on a small scale initial toxicity test, in which, ADN, at doses of 2000, 1000, 500 and 0 mg, was administered by single intragavage dosing (3 mice per group). The mice in the 2000 mg group died within 1 hour after dosing, the mice in 1000 mg group also died within 24 hours, while all the animals in 500 mg group survived the treatment. The PCE/NCE ratio (determined in 1000 cells) in the ADN (500 mg)-exposed animals (1.34) was not different from the control level (1.42). Therefore, a dose of 750 mg was selected as the top dose in the micronuclei study.

1. Toxicity of ADN on Bone Marrow Cell Proliferation

Polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) represent the immature and mature erythrocytes in the bone marrow respectively. The toxicity of the test agents is indicated by the reduction in their ratio. ADN significantly reduced the PCE/NCE ratio in both male and female in a dose-dependent manner ($r = -0.946$, $p<0.005$ in males, and $r = -0.973$, $p<0.005$ in females), as shown in Table 7 and Fig. 7, suggesting the toxic effect of ADN on bone marrow. One way analysis of variance (ANOVA) indicates that there is no significant sex-difference in the bone marrow toxicity of ADN ($F = 0.676$, $p >0.8$).

2. Micronuclei Induction by ADN in Mouse Bone Marrow Cells

The results of the micronuclei induction by ADN are presented in Table 8 and Fig.8. The background frequency of micronucleated cells was 0.34% in males and 0.38% in females (normally between 0.2-0.5%). The positive control, CP, increased the MN frequency by about 7-fold. The test agent, ADN, caused dose-dependent induction of micronuclei ($r = 0.877$, $p < 0.05$ in males, $r = 0.979$, $p < 0.0005$ in females). The maximal induction of micronuclei by ADN (750 mg/kg/dose) was about 3-fold higher than the controls. One way analysis of variance (ANOVA) indicates that there is no significant sex-difference in MN induction by ADN ($F = 0.412$, $p = 0.536$).

3. Conclusion

The above results indicate that under the experimental conditions, ammonium dinitramide significantly increases the micronucleated cell frequency in the Swiss CD-1 mice polychromatic erythrocyte system in a dose-dependent manner, suggesting its chromosome-damage effect in the *in vivo* assay.

Table 1. Mutagenicity Assay of ADN in Ames Test (TA100)

Treatment	Experiment 1		Experiment 2	
	S9-	S9+	S9-	S9+
Control	113	130	109	128
DMSO	111	119	115	128
2-AF (20 ug)	178	1248	149	1168
ADN (0.3125 mg)	101	149	128	139
ADN (0.625 mg)	129	162	143	156
ADN (1.25 mg)	133	199	176	226
ADN (2.5 mg)	197	219	217	285
ADN (5 mg)	240	378	244	372

Table 2. Mutagenicity Assay of ADN in Ames Test (TA98)

Treatment	Experiment 1		Experiment 2	
	S9-	S9+	S9-	S9+
Control	24	36	43	48
DMSO	31	49	44	47
2-AF (20 ug)	134	2197	135	1944
ADN (0.3125 mg)	27	39	37	39
ADN (0.625 mg)	24	41	41	37
ADN (1.25 mg)	28	31	40	41
ADN (2.5 mg)	28	34	45	41
ADN (5 mg)	36	43	46	50

Table 3. Mutagenicity Assay of ADN in Ames Test (TA1535)

Treatment	Experiment 1		Experiment 2	
	S9-	S9+	S9-	S9+
Control	13	17	13	15
DMSO	13	17	14	18
Sodium (2 ug) Azide	110	-	117	-
ADN (0.3125 mg)	15	15	14	16
ADN (0.625 mg)	15	15	15	16
ADN (1.25 mg)	15	18	14	17
ADN (2.5 mg)	14	17	15	15
ADN (5 mg)	15	18	16	17

Table 4. Mutagenicity Assay of ADN in Ames Test (TA1537)

Treatment	Experiment 1		Experiment 2	
	S9-	S9+	S9-	S9+
Control	6	7	6	8
DMSO	7	9	6	9
9-Amino-acridine(10 ug)	39	-	37	-
ADN (0.3125 mg)	6	7	8	7
ADN (0.625 mg)	7	9	7	9
ADN (1.25 mg)	8	7	7	7
ADN (2.5 mg)	8	8	8	8
ADN (5 mg)	8	9	6	8

Table 5. Range-Finding for ADN in Mouse Lymphoma Cell Assay

ADN Treatment (μg/mL)	Daily Growth[a] Day 1	Daily Growth[a] Day 2	Cumulative Cell Count[b] (10^6/mL)	Relative Suspension Growth[c]
Medium	2.53	4.17	3.17	100.0
0.25	3.07	3.90	3.59	113.3
0.5	2.53	4.92	3.74	118.0
2.5	2.37	3.83	2.72	85.9
5	3.08	3.02	2.79	88.1
25	2.67	3.35	2.68	84.6
50	2.63	3.93	3.11	98.1
250	2.63	3.38	2.67	100.0
500	2.60	4.27	3.33	124.5
2500	2.67	3.20	2.56	95.8
5000	1.50	4.02	1.81	67.6

[a] Daily Growth = Observed cell conc./initial seeding conc. (3×10^5cells/mL).
[b] Cumulative Cell Count (CCC) = Initial seeding conc. × Day 1 growth × Day 2 growth.
[c] Relative Suspension Growth = CCC in treatment group/CCC in medium control

Table 6. Mutagenicity of ADN in Mouse Lymphoma cells

Treatment	Experiment 1					Experiment 2				
	VC[a] Mean±SD[f]	TFT[b] Mean±SD	F (10^{-6})[c]	F (Induced)[d]	Relative Frequency[e]	VC[a] Mean±SD	TFT[b] Mean±SD	F (10^{-6})[c]	F (Induced)[d]	Relative Frequency[e]
Medium	274 ± 20.0	70 ± 2.9	51	0	1.00	278 ± 12.3	63 ± 2.1	46	0	1.00
EMS 250 nl/mL	196 ± 8.4**	240 ± 13.2**	244	193	4.78	170 ± 4.7**	220 ± 6.6**	258	212	5.65
50	267 ± 12.4	71 ± 1.9	53	2	1.05	242 ± 10.8*	63 ± 3.4	52	6	1.14
250	275 ± 13.1	65 ± 0.8	47	-4	0.93	209 ± 6.8**	54 ± 3.7	52	6	1.13
500	298 ± 11.4	90 ± 1.4**	60	9	1.18	256 ± 14.5	67 ± 6.0	53	7	1.15
2500	277 ± 15.2	81 ± 2.9*	58	7	1.14	249 ± 23.2	64 ± 3.9	52	6	1.13
5000	280 ± 13.2	99 ± 0.5**	71	19	1.38	240 ± 5.9*	106 ± 7.3**	88	43	1.94
Medium +S9	313 ± 6.2	81 ± 4.5	52	0	1.00	246 ± 3.6	50 ± 6.6	41	0	1.00
3-MCA 2.5 +S9	235 ±20.6**	206 ± 1.4**	175	124	3.40	216 ± 5.0**	151 ± 17.2**	140	99	3.42
50 +S9	310 ± 15.3	92 ± 9.4	59	8	1.15	222 ± 15.5	49 ± 1.2	44	3	1.07
250 +S9	282 ± 9.7*	85 ± 7.4	60	9	1.17	268 ± 9.6	67 ± 5.4*	50	9	1.22
500 +S9	278 ± 10.5*	69 ± 2.2	50	-2	0.96	267 ± 4.8	56 ± 2.9	42	1	1.03
2500 +S9	298 ± 4.9*	87 ± 3.9	59	7	1.14	243 ± 9.4	69 ± 1.2*	57	16	1.40
5000 +S9	356 ± 7.4	210 ± 12.8**	118	67	2.29	193 ± 11.9**	125 ± 8.2**	130	89	3.18

[a] VC = Viable count.
[b] TFT = TFT resistant mutants.
[c] F = Mutation frequency.
[d] F (induced) = F in treatment group - F in medium control.
[e] Relative Frequency = F in treatment group/F in medium control.
[f] Mean ± SD was calculated from triplicate cultures.
*, **: Compared with controls, $p<0.05$ and $p<0.005$.

Table 7. Toxicity of ADN on Mouse Bone Marrow Cell Proliferation (PCE/NCE)

Treatment	Single dose* (mg/kg b.w.)	PCE/NCE	
		Male (Mean±SD)	Female(Mean±SD)
Control	0	1.55 ± 0.01	1.55 ± 0.40
CP	20	0.38 ± 0.07	0.50 ± 0.10
ADN	62.5	1.54 ± 0.17	1.39 ± 0.06
ADN	125	1.16 ± 0.11	1.44 ± 0.19
ADN	250	1.18 ± 0.10	1.26 ± 0.10
ADN	500	1.01 ± 0.09	1.11 ± 0.06
ADN	750	0.65 ± 0.03	0.63 ± 0.05

*ADN was dosed for 3 consecutive days with single daily dose as indicated in the table.

Data for ADN 500 mg/kg in males and ADN 750, 500 and 62.5 mg/kg in females are summarized from 4 mice, while data for other groups are summarized from 5 mice.

Table 8. Micronuclei Induction by ADN in Mouse Bone Marrow Cells

Treatment	Single dose* (mg/kg b.w.)	Micronucleated Cells (%)	
		Male (Mean±SD)	Female(Mean±SD)
Control	0	0.34 ± 0.05	0.38 ± 0.13
CP	20	2.54 ± 0.47	2.62 ± 0.44
ADN	62.5	0.42 ± 0.04	0.30 ± 0.08
ADN	125	0.80 ± 0.19	0.48 ± 0.19
ADN	250	0.70 ± 0.07	0.60 ± 0.07
ADN	500	0.94 ± 0.16	0.85 ± 0.13
ADN	750	1.02 ± 0.19	1.00 ± 0.22

*ADN was dosed for 3 consecutive days with single daily dose as indicated in the table.

Data for ADN 500 mg/kg in males and ADN 750, 500 and 62.5 mg/kg in females are summarized from 4 mice, while data for other groups are summarized from 5 mice.

Fig. 1 Mutagenicity of ADN in TA100

(Experiment A)

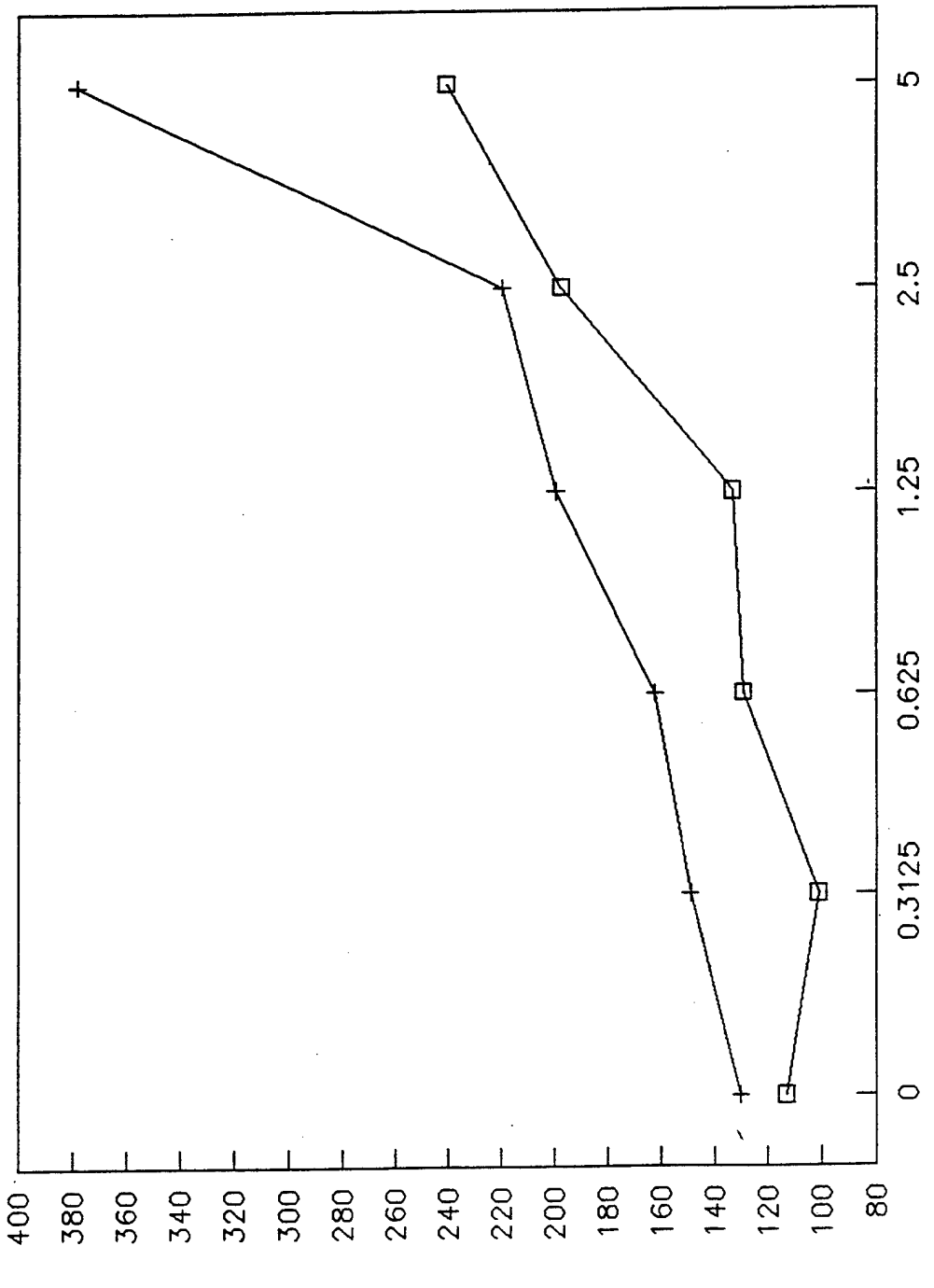


Fig.2 Mutagenicity of ADN in TA100

(Experiment B)

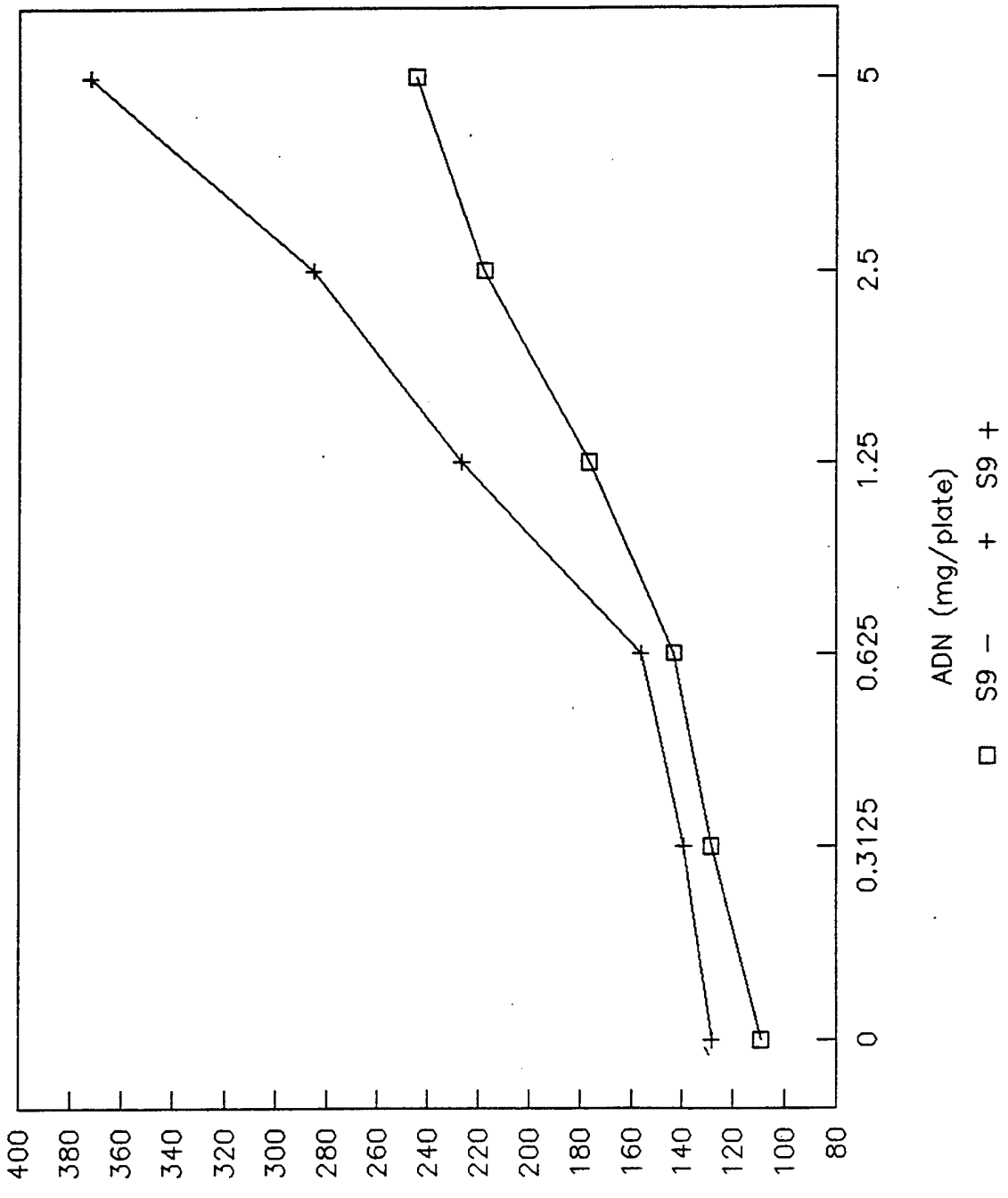


Fig.3 Size Distribution of Mutants

(EMS-induced, S9-)

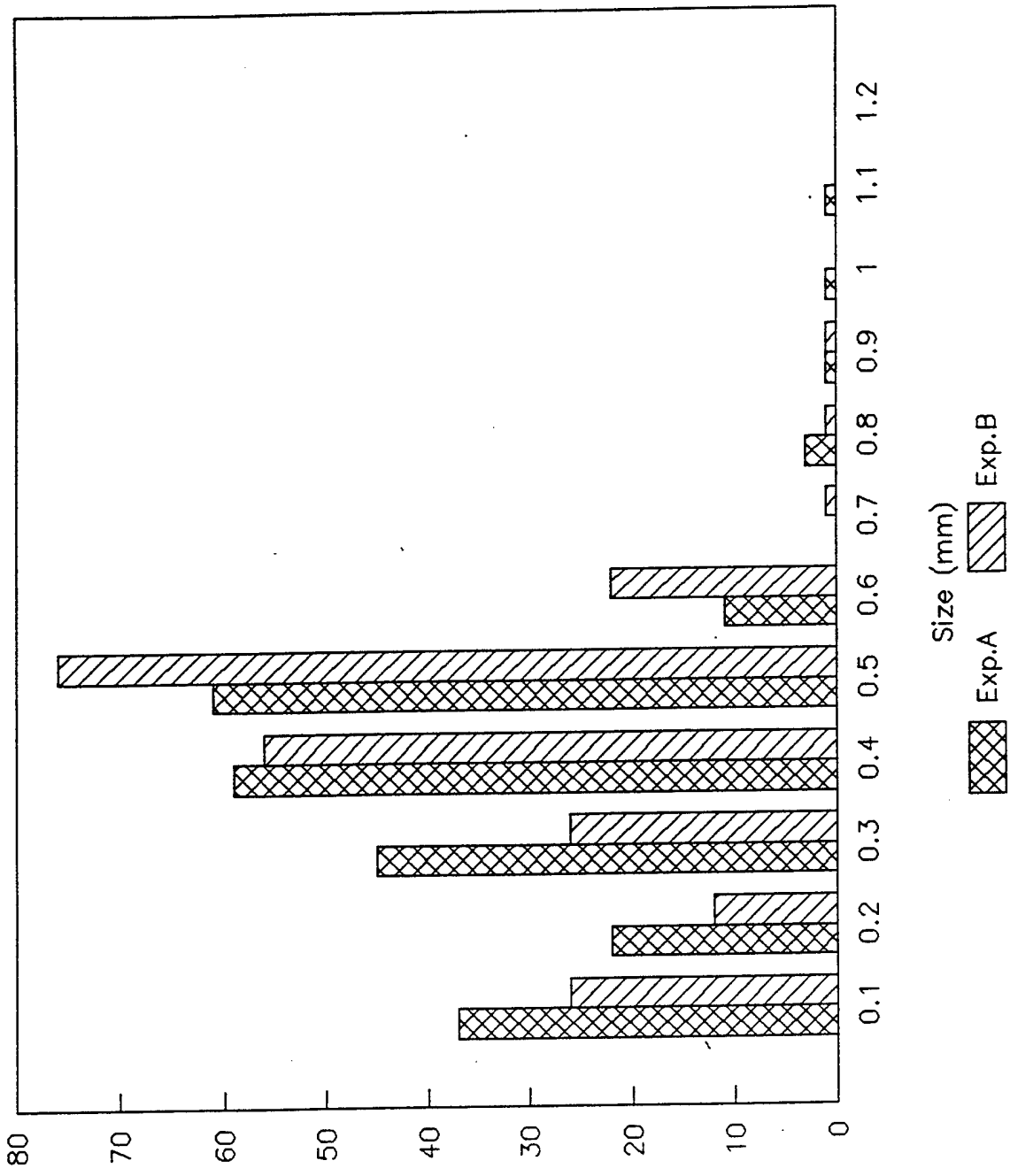


Fig.4 Size Distribution of Mutants

(MCA-induced, S9+)

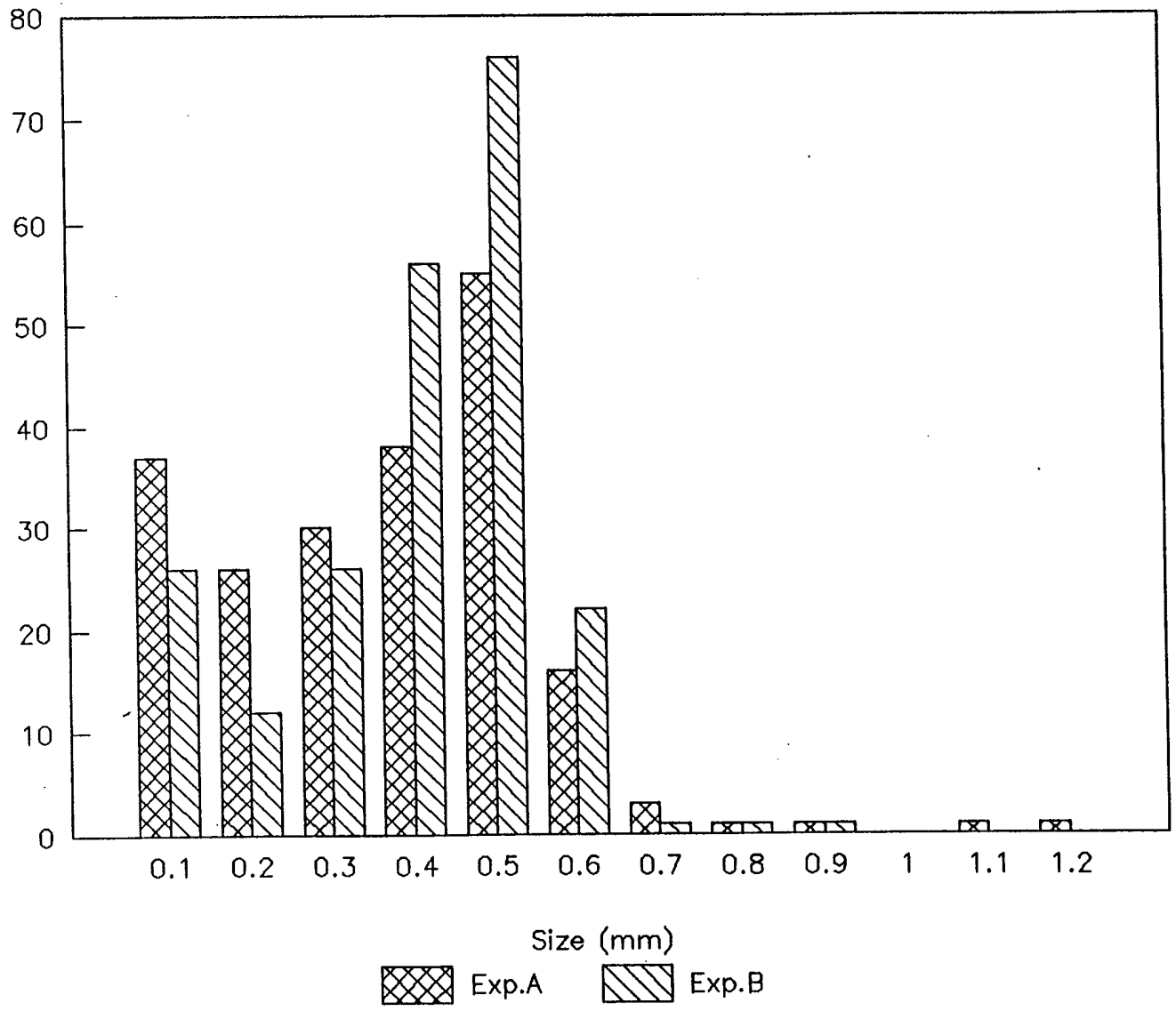


Fig.5 Size Distribution of Mutants

(ADN−induced, S9−)

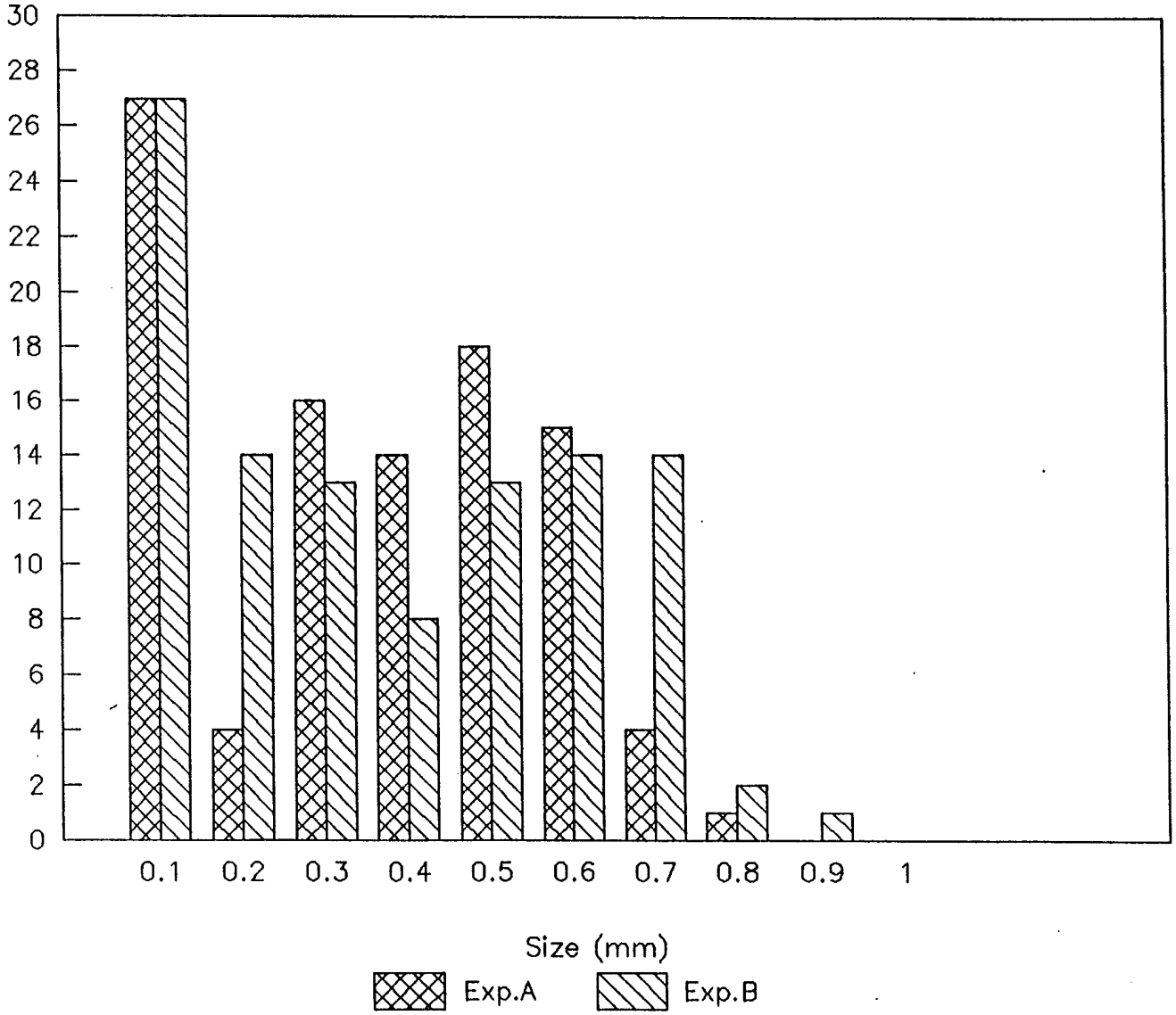


Fig.6 Size Distribution of Mutants

(ADN−induced, S9+)

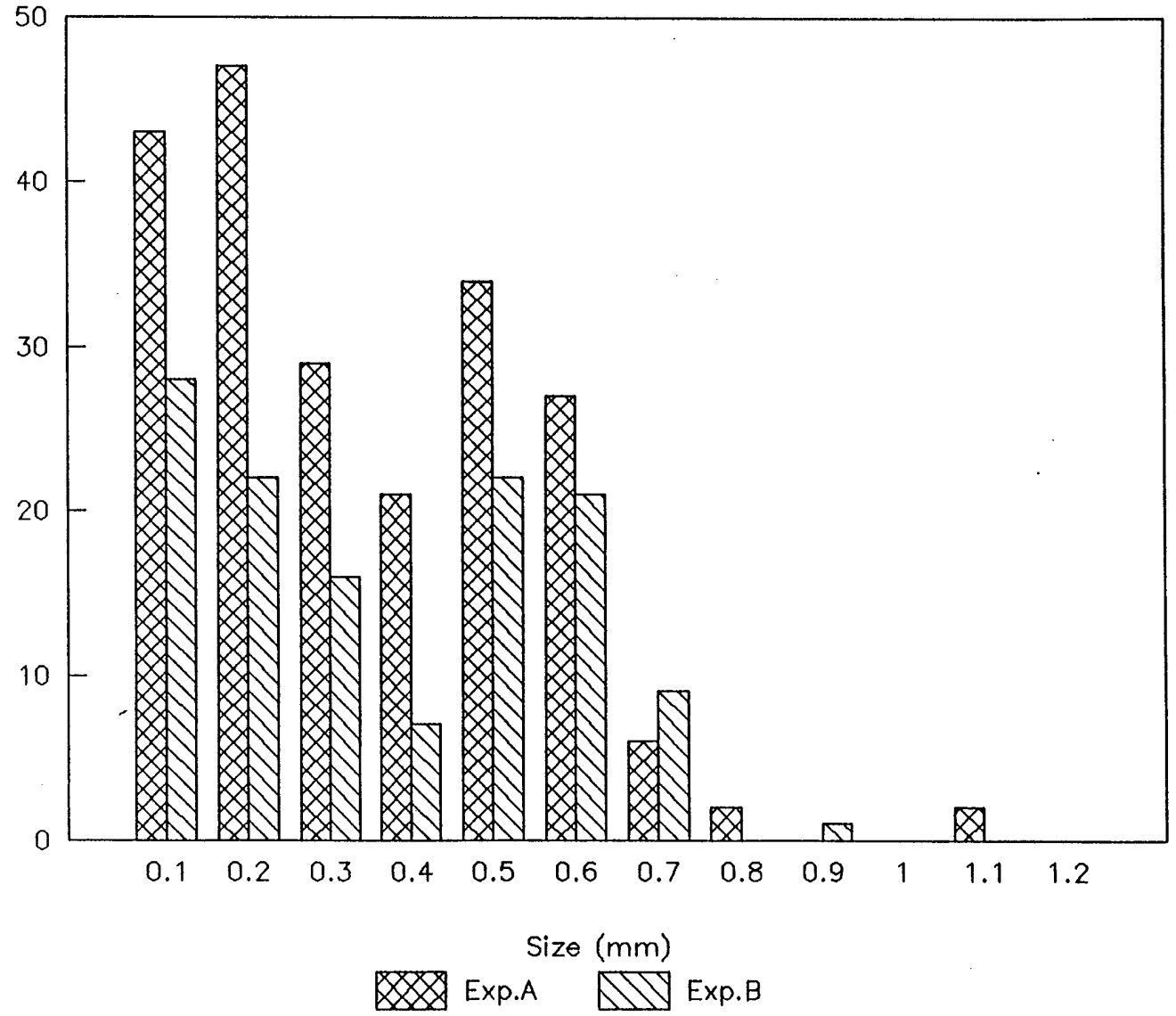


Fig.7 PCE/NCE Changes Induced by ADN

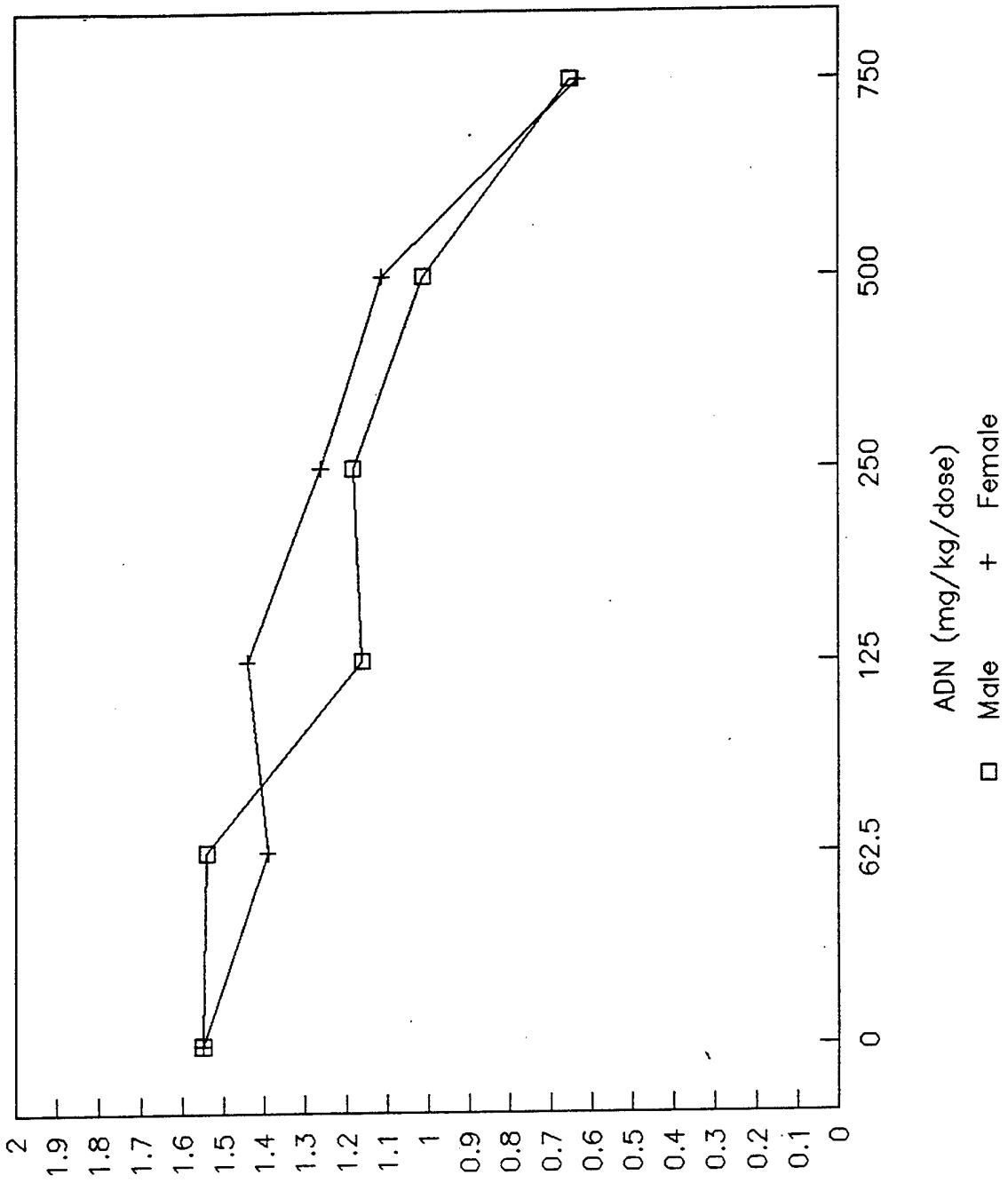
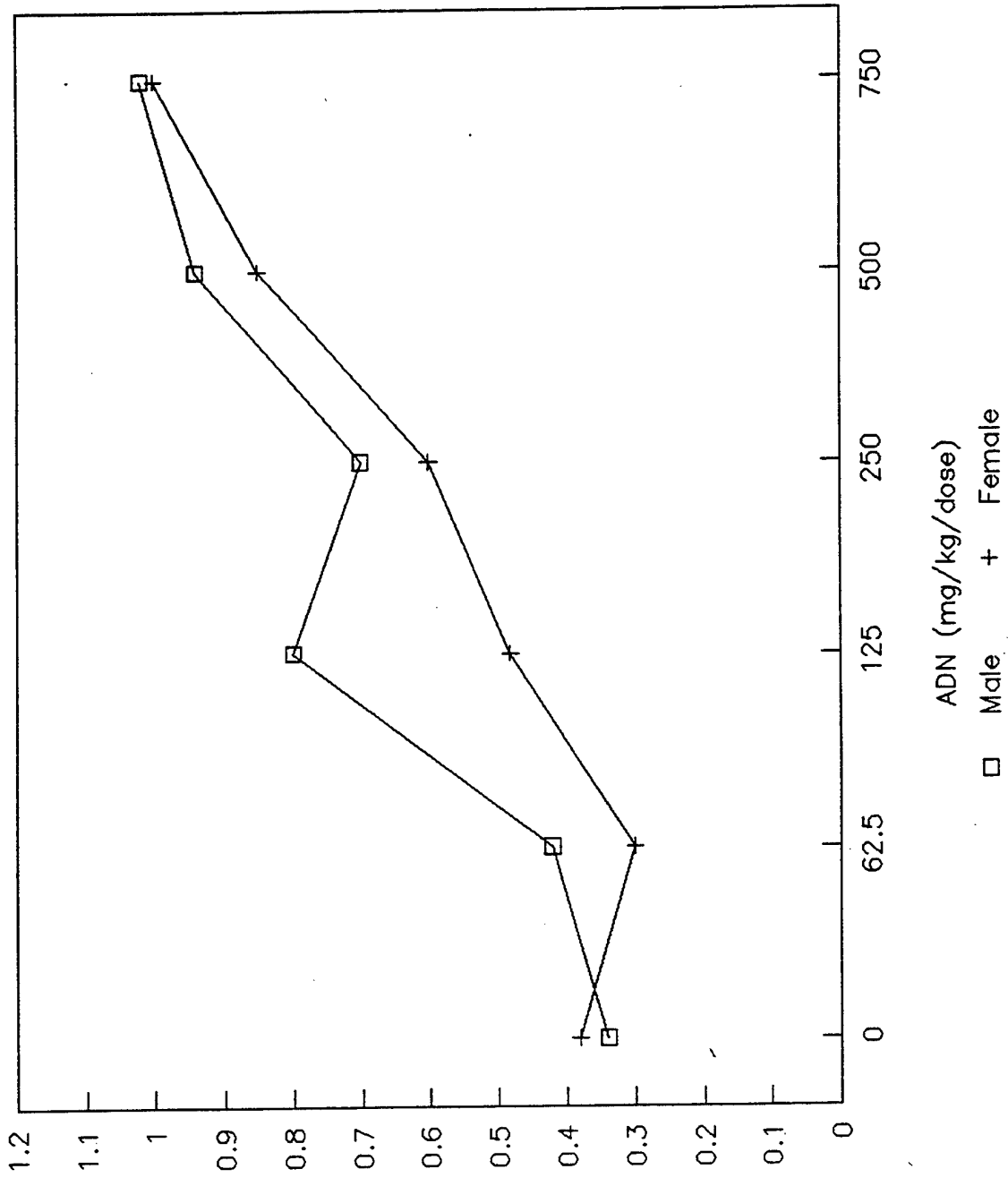


Fig.8 Micronuclei Induced by ADN



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APPENDIX A. Raw Data of Salmonella/microsome Mutagenesis

Appendix–A1. Ames Test – TA100
Exp. 060194
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		131.0	136.0	141.0	136.0	112.7	16.6	1.0
		101.0	98.0	99.0	99.3			
		96.0	103.0	109.0	102.7			
DMSO		114.0	110.0	112.0	112.0	111.4	2.3	1.0
		107.0	107.0	111.0	108.3			
		113.0	117.0	112.0	114.0			
2–AF	20 ug	175.0	180.0	184.0	179.7	177.8	5.2	1.6
		188.0	179.0	182.0	183.0			
		168.0	170.0	174.0	170.7			
ADN	5 mg	215.0	218.0	240.0	224.3	240.2	20.2	2.1
		225.0	223.0	235.0	227.7			
		270.0	268.0	268.0	268.7			
ADN	2.5 mg	190.0	188.0	184.0	187.3	196.7	12.0	1.7
		191.0	188.0	188.0	189.0			
		220.0	206.0	215.0	213.7			
ADN	1.25 mg	140.0	139.0	145.0	141.3	132.6	8.5	1.2
		135.0	136.0	135.0	135.3			
		119.0	121.0	123.0	121.0			
ADN	0.625 mg	108.0	110.0	118.0	112.0	128.6	12.6	1.1
		130.0	131.0	132.0	131.0			
		141.0	144.0	143.0	142.7			
ADN	0.3125 mg	100.0	103.0	100.0	101.0	101.3	1.5	0.9
		101.0	99.0	99.0	99.7			
		103.0	103.0	104.0	103.3			
S9 (+)								
Control		132.0	131.0	133.0	132.0	130.2	10.1	1.0
		117.0	116.0	118.0	117.0			
		143.0	140.0	142.0	141.7			
DMSO		103.0	108.0	108.0	106.3	119.2	9.3	0.9
		121.0	125.0	125.0	123.7			
		129.0	129.0	125.0	127.7			
2–AF	20 ug	1235.0	1278.0	1225.0	1246.0	1247.8	1.3	9.6
		1262.0	1265.0	1219.0	1248.7			
		1233.0	1268.0	1245.0	1248.7			
ADN	5 mg	354.0	349.0	352.0	351.7	378.1	18.9	2.9
		391.0	394.0	398.0	394.3			
		386.0	389.0	390.0	388.3			
ADN	2.5 mg	206.0	223.0	214.0	214.3	219.4	7.2	1.7
		214.0	212.0	217.0	214.3			
		223.0	231.0	235.0	229.7			
ADN	1.25 mg	221.0	216.0	215.0	217.3	198.9	13.2	1.5
		190.0	185.0	186.0	187.0			
		196.0	190.0	191.0	192.3			
ADN	0.625 mg	168.0	163.0	165.0	165.3	162.4	9.2	1.2
		167.0	172.0	177.0	172.0			
		143.0	146.0	161.0	150.0			
ADN	0.3125 mg	142.0	139.0	147.0	142.7	148.6	7.9	1.1
		158.0	162.0	159.0	159.7			
		144.0	143.0	143.0	143.3			

Appendix–A2. Ames Test – TA100
Exp. 060894
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		117.0	112.0	107.0	112.0	109.2	8.3	1.0
		114.0	118.0	121.0	117.7			
		98.0	94.0	102.0	98.0			
DMSO		115.0	112.0	118.0	115.0	115.0	6.0	1.1
		107.0	102.0	114.0	107.7			
		125.0	124.0	118.0	122.3			
2–AF	20 ug	149.0	152.0	150.0	150.3	147.8	10.4	1.4
		155.0	162.0	160.0	159.0			
		147.0	150.0	155.0	134.0			
ADN	5 mg	260.0	247.0	281.0	262.7	244.1	14.7	2.2
		220.0	225.0	235.0	226.7			
		235.0	245.0	249.0	243.0			
ADN	2.5 mg	212.0	217.0	226.0	218.3	216.6	4.8	2.0
		212.0	209.0	209.0	210.0			
		221.0	216.0	227.0	221.3			
ADN	1.25 mg	185.0	183.0	184.0	184.0	176.2	6.7	1.6
		176.0	165.0	162.0	167.7			
		182.0	176.0	173.0	177.0			
ADN	0.625 mg	145.0	157.0	143.0	148.3	142.9	9.4	1.3
		133.0	126.0	130.0	129.7			
		148.0	157.0	147.0	150.7			
ADN	0.3125 mg	109.0	117.0	120.0	115.3	127.8	9.3	1.2
		143.0	138.0	132.0	137.7			
		136.0	131.0	124.0	130.3			
S9 (+)								
Control		127.0	119.0	118.0	121.3	128.0	6.4	1.0
		126.0	127.0	125.0	126.0			
		158.0	124.0	128.0	136.7			
DMSO		126.0	129.0	123.0	126.0	127.7	1.4	1.0
		133.0	126.0	129.0	129.3			
		127.0	132.0	124.0	127.7			
2–AF	20 ug	1145.0	1158.0	1235.0	1179.3	1167.7	31.4	9.1
		1175.0	1187.0	1235.0	1199.0			
		1074.0	1158.0	1142.0	1124.7			
ADN	5 mg	396.0	399.0	405.0	400.0	371.7	20.0	2.9
		372.0	338.0	365.0	358.3			
		345.0	358.0	367.0	356.7			
ADN	2.5 mg	306.0	300.0	305.0	303.7	285.1	16.6	2.2
		307.0	282.0	276.0	288.3			
		268.0	245.0	277.0	263.3			
ADN	1.25 mg	209.0	228.0	232.0	223.0	225.9	2.1	1.8
		219.0	227.0	235.0	227.0			
		223.0	232.0	228.0	227.7			
ADN	0.625 mg	156.0	158.0	168.0	160.7	155.7	3.7	1.2
		154.0	148.0	154.0	152.0			
		159.0	150.0	154.0	154.3			
ADN	0.3125 mg	148.0	142.0	138.0	142.7	139.0	5.2	1.1
		137.0	142.0	149.0	142.7			
		122.0	132.0	141.0	131.7			

Appendix–A3. Ames Test – TA98
Exp. 060194
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		25.0	21.0	23.0	23.0	24.0	5.6	1.0
		18.0	17.0	18.0	17.7			
		33.0	32.0	29.0	31.3			
DMSO		23.0	24.0	24.0	23.7	30.7	5.9	1.3
		32.0	29.0	30.0	30.3			
		37.0	38.0	39.0	38.0			
2–AF	20 ug	161.0	154.0	144.0	153.0	134.0	13.9	5.6
		130.0	136.0	121.0	129.0			
		135.0	117.0	108.0	120.0			
ADN	5 mg	31.0	35.0	32.0	32.7	36.2	2.5	1.5
		37.0	39.0	37.0	37.7			
		38.0	38.0	39.0	38.3			
ADN	2.5 mg	24.0	25.0	25.0	24.7	27.9	2.5	1.2
		30.0	27.0	28.0	28.3			
		30.0	33.0	29.0	30.7			
ADN	1.25 mg	29.0	31.0	31.0	30.3	27.9	1.9	1.2
		30.0	25.0	28.0	27.7			
		25.0	26.0	26.0	25.7			
ADN	0.625 mg	25.0	24.0	25.0	24.7	23.8	1.5	1.0
		27.0	25.0	23.0	25.0			
		19.0	22.0	24.0	21.7			
ADN	0.3125 mg	27.0	28.0	27.0	27.3	26.8	1.8	1.1
		31.0	27.0	28.0	28.7			
		25.0	24.0	24.0	24.3			
S9 (+)								
Control		38.0	35.0	33.0	35.3	36.0	0.5	1.0
		36.0	35.0	36.0	36.3			
		37.0	36.0	36.0	36.3			
DMSO		58.0	58.0	58.0	58.0	48.8	6.6	1.4
		41.0	48.0	46.0	45.0			
		43.0	45.0	42.0	43.3			
2–AF	20 ug	2078.0	2154.0	2136.0	2122.7	2196.9	173.3	61.0
		2078.0	1985.0	2032.0	2031.7			
		2443.0	2436.0	2430.0	2436.3			
ADN	5 mg	54.0	53.0	54.0	53.7	43.1	7.5	1.2
		37.0	36.0	40.0	37.7			
		35.0	40.0	39.0	38.0			
ADN	2.5 mg	30.0	32.0	31.0	31.0	34.1	2.5	0.9
		34.0	35.0	34.0	34.3			
		36.0	38.0	37.0	37.0			
ADN	1.25 mg	28.0	31.0	34.0	31.0	30.9	0.2	0.9
		31.0	29.0	32.0	30.7			
		28.0	32.0	33.0	31.0			
ADN	0.625 mg	38.0	38.0	42.0	39.3	40.7	3.1	1.1
		46.0	44.0	45.0	45.0			
		38.0	37.0	38.0	37.7			
ADN	0.3125 mg	38.0	41.0	42.0	40.3	39.4	2.8	1.1
		43.0	43.0	41.0	42.3			
		34.0	35.0	38.0	35.7			

Appendix–A4. Ames Test – TA98
Exp. 060894
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		39.0	37.0	42.0	39.3	42.6	2.9	1.0
		42.0	43.0	41.0	42.0			
		46.0	47.0	46.0	46.3			
DMSO		39.0	42.0	35.0	38.7	44.2	6.1	1.0
		56.0	54.0	48.0	52.7			
		37.0	42.0	45.0	41.3			
2–AF	20 ug	135.0	138.0	132.0	135.0	134.6	8.4	3.2
		117.0	120.0	135.0	124.0			
		154.0	136.0	144.0	144.7			
ADN	5 mg	47.0	45.0	42.0	44.7	46.1	4.3	1.1
		38.0	46.0	41.0	41.7			
		49.0	52.0	55.0	52.0			
ADN	2.5 mg	44.0	48.0	47.0	46.3	45.4	1.3	1.1
		45.0	46.0	48.0	46.3			
		39.0	45.0	47.0	43.7			
ADN	1.25 mg	39.0	41.0	44.0	41.3	40.1	2.0	0.9
		38.0	45.0	42.0	41.7			
		35.0	38.0	39.0	37.3			
ADN	0.625 mg	35.0	44.0	45.0	41.3	40.7	1.7	1.0
		47.0	42.0	38.0	42.3			
		39.0	32.0	44.0	38.3			
ADN	0.3125 mg	36.0	35.0	38.0	36.3	36.7	1.0	0.9
		39.0	37.0	38.0	38.0			
		35.0	34.0	38.0	35.7			
S9 (+)								
Control		42.0	39.0	39.0	40.0	48.3	6.4	1.0
		47.0	52.0	49.0	49.3			
		57.0	54.0	56.0	55.7			
DMSO		43.0	44.0	42.0	43.0	46.7	5.2	1.0
		54.0	56.0	52.0	54.0			
		40.0	45.0	44.0	43.0			
2–AF	20 ug	1987.0	1895.0	1954.0	1945.3	1944.4	18.0	40.2
		1899.0	1944.0	1923.0	1922.0			
		1966.0	1978.0	1954.0	1966.0			
ADN	5 mg	58.0	54.0	56.0	56.0	50.1	4.8	1.0
		47.0	44.0	42.0	44.3			
		47.0	51.0	52.0	50.0			
ADN	2.5 mg	46.0	47.0	44.0	45.7	41.0	3.5	0.8
		38.0	35.0	39.0	37.3			
		37.0	42.0	41.0	40.0			
ADN	1.25 mg	34.0	38.0	39.0	37.0	40.7	2.6	0.8
		45.0	42.0	41.0	42.7			
		43.0	40.0	44.0	42.3			
ADN	0.625 mg	40.0	40.0	38.0	39.3	37.2	1.8	0.8
		36.0	37.0	39.0	37.3			
		32.0	37.0	36.0	35.0			
ADN	0.3125 mg	40.0	44.0	42.0	42.0	38.9	2.3	0.8
		41.0	35.0	38.0	38.0			
		35.0	38.0	37.0	36.7			

Appendix–A5. Ames Test – TA1535
Exp. 060194
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		16.0	15.0	13.0	14.7	12.7	1.5	1.0
		12.0	10.0	11.0	11.0			
		14.0	12.0	11.0	12.3			
DMSO		10.0	14.0	14.0	12.7	13.3	1.4	1.1
		12.0	11.0	13.0	12.0			
		15.0	17.0	14.0	15.3			
Sodium Azide	2 ug	115.0	104.0	98.0	105.7	110.2	5.3	8.7
		121.0	118.0	114.0	117.7			
		119.0	104.0	99.0	107.3			
ADN	5 mg	17.0	17.0	15.0	16.3	15.0	1.1	1.2
		16.0	15.0	14.0	15.0			
		12.0	14.0	15.0	13.7			
ADN	2.5 mg	13.0	13.0	14.0	13.3	13.8	1.1	1.1
		14.0	15.0	17.0	15.3			
		10.0	12.0	16.0	12.7			
ADN	1.25 mg	18.0	15.0	14.0	15.7	15.1	0.6	1.2
		17.0	15.0	14.0	15.3			
		12.0	17.0	14.0	14.3			
ADN	0.625 mg	13.0	14.0	15.0	14.0	14.9	1.3	1.2
		16.0	15.0	11.0	14.0			
		18.0	15.0	17.0	16.7			
ADN	0.3125 mg	13.0	12.0	15.0	13.3	15.2	1.4	1.2
		16.0	17.0	17.0	16.7			
		18.0	15.0	14.0	15.7			
S9 (+)								
Control		17.0	18.0	17.0	17.3	17.0	2.1	1.0
		16.0	14.0	13.0	14.3			
		18.0	19.0	21.0	19.3			
DMSO		21.0	18.0	19.0	19.3	17.2	1.5	1.0
		14.0	16.0	17.0	15.7			
		15.0	17.0	18.0	16.7			
				0.0				
ADN	5 mg	21.0	22.0	18.0	20.3	18.2	1.5	1.1
		18.0	17.0	16.0	17.0			
		15.0	18.0	19.0	17.3			
ADN	2.5 mg	18.0	17.0	15.0	16.7	17.0	1.5	1.0
		15.0	14.0	17.0	15.3			
		19.0	17.0	21.0	19.0			
ADN	1.25 mg	22.0	20.0	19.0	20.3	17.6	2.0	1.0
		14.0	17.0	17.0	16.0			
		15.0	17.0	17.0	16.3			
ADN	0.625 mg	11.0	14.0	15.0	13.3	14.8	1.2	0.9
		14.0	15.0	15.0	14.7			
		18.0	16.0	15.0	16.3			
ADN	0.3125 mg	17.0	16.0	14.0	15.7	15.3	0.5	0.9
		15.0	15.0	17.0	15.7			
		14.0	17.0	13.0	14.7			

Appendix–A6. Ames Test – TA1535
Exp. 060894
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		15.0	12.0	13.0	13.3	13.3	0.5	1.0
		13.0	11.0	14.0	12.7			
		14.0	12.0	16.0	14.0			
DMSO		11.0	16.0	14.0	13.7	14.1	0.6	1.1
		14.0	15.0	16.0	15.0			
		12.0	14.0	15.0	13.7			
Sodium Azide	2 ug	106.0	114.0	97.0	105.7	117.1	11.8	8.8
		131.0	128.0	141.0	133.3			
		123.0	110.0	104.0	112.3			
ADN	5 mg	12.0	15.0	16.0	14.3	15.8	1.0	1.2
		15.0	17.0	17.0	16.3			
		15.0	17.0	18.0	16.7			
ADN	2.5 mg	15.0	14.0	13.0	14.0	14.8	1.6	1.1
		17.0	15.0	19.0	17.0			
		12.0	13.0	15.0	13.3			
ADN	1.25 mg	12.0	14.0	16.0	14.0	13.6	1.9	1.0
		11.0	10.0	12.0	11.0			
		18.0	15.0	14.0	15.7			
ADN	0.625 mg	15.0	14.0	16.0	15.0	14.7	1.0	1.1
		12.0	13.0	15.0	13.3			
		14.0	16.0	17.0	15.7			
ADN	0.3125 mg	15.0	12.0	13.0	13.3	14.2	1.0	1.1
		14.0	16.0	17.0	15.7			
		12.0	14.0	15.0	13.7			
S9 (+)								
Control		12.0	15.0	15.0	14.0	15.2	1.0	1.0
		14.0	17.0	15.0	15.3			
		15.0	17.0	17.0	16.3			
DMSO		16.0	15.0	17.0	16.0	17.6	1.5	1.2
		18.0	17.0	16.0	17.0			
		20.0	21.0	18.0	19.7			
ADN	5 mg	22.0	19.0	18.0	19.7	17.2	1.7	1.1
		17.0	16.0	14.0	15.7			
		17.0	17.0	15.0	16.3			
ADN	2.5 mg	15.0	14.0	14.0	14.3	14.8	1.1	1.0
		14.0	15.0	12.0	13.7			
		18.0	15.0	16.0	16.3			
ADN	1.25 mg	21.0	18.0	19.0	19.3	17.0	2.2	1.1
		14.0	15.0	13.0	14.0			
		20.0	17.0	16.0	17.7			
ADN	0.625 mg	12.0	14.0	14.0	13.3	15.6	2.1	1.0
		15.0	16.0	14.0	15.0			
		20.0	18.0	17.0	18.3			
ADN	0.3125 mg	19.0	17.0	17.0	17.7	16.3	1.1	1.1
		16.0	14.0	15.0	15.0			
		15.0	17.0	17.0	16.3			

Appendix–A7. Ames Test – TA1537
Exp. 060194
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		6.0	5.0	6.0	5.7	6.4	0.6	1.0
		5.0	8.0	7.0	6.7			
		6.0	8.0	7.0	7.0			
DMSO		10.0	10.0	11.0	10.3	7.3	2.1	1.1
		8.0	5.0	5.0	6.0			
		4.0	7.0	6.0	5.7			
9–AA	10 ug	35.0	32.0	34.0	33.7	38.8	3.7	5.6
		41.0	44.0	42.0	42.3			
		42.0	40.0	39.0	40.3			
ADN	5 mg	6.0	7.0	8.0	7.0	7.8	0.9	1.2
		9.0	10.0	8.0	9.0			
		8.0	7.0	7.0	7.3			
ADN	2.5 mg	6.0	6.0	5.0	5.7	7.9	1.8	1.2
		11.0	10.0	9.0	10.0			
		7.0	9.0	8.0	8.0			
ADN	1.25 mg	7.0	6.0	6.0	6.3	7.8	1.1	1.2
		10.0	9.0	8.0	9.0			
		9.0	8.0	7.0	8.0			
ADN	0.625 mg	6.0	8.0	7.0	7.0	6.7	0.3	1.0
		7.0	6.0	6.0	6.3			
		8.0	5.0	7.0	6.7			
ADN	0.3125 mg	6.0	5.0	7.0	6.0	6.2	0.3	1.0
		5.0	5.0	8.0	6.0			
		8.0	7.0	5.0	6.7			
S9 (+)								
Control		6.0	5.0	7.0	6.0	6.9	1.0	1.0
		8.0	6.0	5.0	6.3			
		10.0	8.0	7.0	8.3			
DMSO		10.0	9.0	8.0	9.0	9.0	0.3	1.3
		11.0	9.0	8.0	9.3			
		10.0	8.0	8.0	8.7			
ADN	5 mg	10.0	11.0	9.0	10.0	9.0	1.0	1.3
		8.0	9.0	11.0	9.3			
		7.0	7.0	9.0	7.7			
ADN	2.5 mg	11.0	10.0	8.0	9.7	8.4	1.3	1.2
		10.0	9.0	8.0	9.0			
		6.0	7.0	7.0	6.7			
ADN	1.25 mg	8.0	7.0	7.0	7.3	7.0	0.5	1.0
		8.0	7.0	7.0	7.3			
		6.0	7.0	6.0	6.3			
ADN	0.625 mg	9.0	9.0	8.0	8.7	9.1	0.6	1.3
		9.0	9.0	8.0	8.7			
		11.0	10.0	9.0	10.0			
ADN	0.3125 mg	8.0	7.0	7.0	7.3	6.9	0.6	1.0
		6.0	5.0	7.0	6.0			
		6.0	8.0	8.0	7.3			

Appendix–A8. Ames Test – TA1537
Exp. 060894
Study #: 1093–A06–1

Agent	Dose	Count 1	Count 2	Count 3	Mean(1)	Mean(2)	SD	Ratio
S9 (–):								
Control		7.0	8.0	8.0	7.7	6.4	1.0	1.0
		5.0	5.0	6.0	5.3			
		6.0	8.0	5.0	6.3			
DMSO		7.0	6.0	6.0	6.3	5.8	0.6	0.9
		5.0	4.0	6.0	5.0			
		7.0	5.0	6.0	6.0			
9–AA	10 ug	32.0	37.0	35.0	34.7	37.1	2.4	4.5
		38.0	35.0	36.0	36.3			
		39.0	42.0	40.0	40.3			
ADN	5 mg	6.0	5.0	5.0	5.3	6.4	1.0	1.0
		7.0	8.0	8.0	7.7			
		6.0	7.0	6.0	6.3			
ADN	2.5 mg	8.0	9.0	9.0	8.7	7.9	1.1	1.2
		10.0	8.0	8.0	8.7			
		7.0	6.0	6.0	6.3			
ADN	1.25 mg	10.0	8.0	8.0	8.7	6.8	1.5	1.1
		8.0	6.0	6.0	6.7			
		5.0	4.0	6.0	5.0			
ADN	0.625 mg	5.0	5.0	7.0	5.7	7.2	1.2	1.1
		8.0	8.0	6.0	7.3			
		7.0	9.0	10.0	8.7			
ADN	0.3125 mg	10.0	8.0	8.0	8.7	7.7	0.7	1.2
		8.0	7.0	6.0	7.0			
		7.0	7.0	8.0	7.3			
S9 (+)								
Control		8.0	8.0	9.0	8.3	8.2	0.7	1.0
		7.0	8.0	7.0	7.3			
		10.0	9.0	8.0	9.0			
DMSO		9.0	9.0	7.0	8.3	9.0	0.9	1.1
		8.0	9.0	8.0	8.3			
		10.0	11.0	10.0	10.3			
ADN	5 mg	8.0	7.0	8.0	7.7	8.0	1.8	1.0
		10.0	11.0	10.0	10.3			
		7.0	5.0	6.0	6.0			
ADN	2.5 mg	8.0	8.0	7.0	7.7	7.6	0.4	0.9
		6.0	7.0	8.0	7.0			
		8.0	8.0	8.0	8.0			
ADN	1.25 mg	7.0	7.0	9.0	7.7	7.3	1.5	0.9
		6.0	5.0	5.0	5.3			
		8.0	9.0	10.0	9.0			
ADN	0.625 mg	11.0	10.0	9.0	10.0	8.7	1.1	1.1
		8.0	7.0	7.0	7.3			
		10.0	8.0	8.0	8.7			
ADN	0.3125 mg	6.0	7.0	7.0	6.7	6.7	0.3	0.8
		8.0	7.0	6.0	7.0			
		5.0	7.0	7.0	6.3			

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APPENDIX B. Raw Data of Mouse Lymphoma cell Mutagenesis

Appendix-B1. Raw Data Record for the Range-Finding Experiment with ADN (Study #:1093-A06-2)

Treatment (μg/mL)	Day 1			Day 2		
	Cell Counts	Cell Conc. (10^6/mL)	Daily Growth[a]	Cell Counts	Cell Conc. (10^6/mL)	Daily Growth[a]
Medium	74 78	0.760	2.53	116 134	1.250	4.17
0.25	102 82	0.920	30.7	113 121	1.170	3.90
0.5	76 76	0.760	2.53	162 133	1.475	4.92
2.5	69 73	0.710	2.37	102 128	1.150	3.83
5	98 87	0.925	3.08	81 100	0.905	3.02
25	77 83	0.800	2.67	116 85	1.005	3.35
50	86 72	0.790	2.63	113 123	1.180	3.93
250	81 77	0.790	2.63	94 109	1.015	3.38
500	82 74	0.780	2.60	129 127	1.280	4.27
2500	76 84	0.800	2.67	95 97	0.960	3.20
5000	43 47	0.450	1.50	131 110	1.205	4.02

[a] Daily Growth = Cell conc./Initial seeding conc. (3×10^5/mL)

Appendix-B2. Raw Data Record for the Mutagenesis Assay Experiment A without S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Day 1 Cell Counts	Day 1 Cell Conc. (10^6/mL)	Day 1 Daily Growth	Day 2 Cell Counts	Day 2 Cell Conc. (10^6/mL)	Day 2 Daily Growth	Relative Suspension Growth[a]	Relative Plating Efficiency[b]	Relative Total Growth[c]	Viable Counts	TFT Mutant Counts
Medium	110 87	0.985	3.28	111 107	1.090	3.63	100.0	100.0	100.0	249 298 275	67 69 74
EMS 250 nl/mL	103 81	0.920	3.17	106 94	1.000	3.33	85.7	72.7	61.4	205 199 185	257 225 237
50	118 128	1.230	4.10	100 96	0.980	3.27	112.3	97.5	109.4	279 250 272	70 70 74
250	83 109	0.960	3.20	108 114	1.110	3.70	99.3	100.4	99.6	262 270 293	64 66 65
500	105 97	1.010	3.37	96 106	1.010	3.37	95.0	108.6	103.2	282 302 309	89 92 89
2500	96 87	0.900	3.00	124 99	1.115	3.72	93.5	101.2	94.6	275 260 297	77 84 81
5000	65 61	0.630	2.10	106 103	1.045	3.48	61.3	102.1	62.6	297 265 277	99 99 98

[a] Relative Suspension Growth = Cumulative cell count (CCC) in treatment group/ CCC in medium control.
[b] Relative Plating Efficiency = Mean number of VC colonies in treatment group/ Mean number of VC colonies in solvent control.
[c] Relative Total Growth = Day 2 Relative % Suspension Growth × Relative Plating Efficiency.

Appendix-B3. Raw Data Record for the Mutagenesis Assay Experiment A with S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Day 1 Cell Counts	Day 1 Cell Conc. (10^6/mL)	Day 1 Daily Growth	Day 2 Cell Counts	Day 2 Cell Conc. (10^6/mL)	Day 2 Daily Growth	Relative Suspension Growth[a]	Relative Plating Efficiency[b]	Relative Total Growth[c]	Viable Counts	TFT Mutant Counts
Medium +S9	101 117	1.090	3.63	97 101	0.990	3.30	100.0	100.0	100.0	321 312 306	75 86 81
3-MCA 2.5 +S9	64 78	0.710	2.37	140 139	1.395	4.65	91.8	75.01	68.9	212 231 262	205 208 205
50 +S9	86 84	0.850	2.83	106 116	1.110	3.70	87.4	99.2	86.7	327 290 314	92 80 103
250 +S9	115 103	1.090	3.63	95 93	0.940	3.13	94.9	90.0	85.4	287 290 268	76 94 84
500 +S9	97 107	1.020	3.40	112 112	1.120	3.73	105.9	88.7	93.9	287 263 283	72 68 67
2500 +S9	92 77	0.845	2.82	106 115	1.105	3.68	86.5	95.3	82.5	299 292 304	89 82 91
5000 +S9	60 60	0.600	2.00	69 53	0.610	2.03	33.9	113.6	38.5	364 357 346	192 221 217

[a] Relative Suspension Growth = Cumulative cell count (CCC) in treatment group/ CCC in medium control.
[b] Relative Plating Efficiency = Mean number of VC colonies in treatment group/ Mean number of VC colonies in solvent control.
[c] Relative Total Growth = Day 2 Relative % Suspension Growth × Relative Plating Efficiency.

Appendix-B4. Raw Data Record for the Mutagenesis Assay Experiment B without S9 Activation (Study #:1093-A06-2)

	Day 1			Day 2							
Treatment (μg/mL)	Cell Counts	Cell Conc. (10^6/mL)	Daily Growth	Cell Counts	Cell Conc. (10^6/mL)	Daily Growth	Relative Suspension Growth[a]	Relative Plating Efficiency[b]	Relative Total Growth[c]	Viable Counts	TFT Mutant Counts
Medium	76 69	0.710	2.37	117 94	1.055	3.52	100.0	100.0	100.0	293 277 263	63 61 66
EMS 250 nl/mL	83 87	0.850	2.83	108 92	1.000	3.33	113.5	61.3	69.6	167 177 167	229 216 214
50	106 92	0.990	3.30	121 101	1.110	3.70	146.7	87.2	127.9	252 247 227	66 58 64
250	120 134	1.270	4.23	102 111	1.065	3.55	180.6	75.4	136.1	212 200 216	55 58 49
500	104 116	1.100	3.67	107 107	1.070	3.57	157.1	92.3	145.1	260 237 272	59 73 70
2500	87 96	0.915	3.05	118 104	1.110	3.70	135.6	89.8	121.8	236 282 230	68 59 66
5000	66 74	0.700	2.33	114 106	1.100	3.67	102.8	86.4	88.9	238 248 234	109 113 96

[a] Relative Suspension Growth = Cumulative cell count (CCC) in treatment group/ CCC in medium control.
[b] Relative Plating Efficiency = Mean number of VC colonies in treatment group/ Mean number of VC colonies in solvent control.
[c] Relative Total Growth = Day 2 Relative % Suspension Growth × Relative Plating Efficiency.

Appendix-B5. Raw Data Record for the Mutagenesis Assay Experiment B with S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Day 1 Cell Counts	Day 1 Cell Conc. (10^6/mL)	Day 1 Daily Growth	Day 2 Cell Counts	Day 2 Cell Conc. (10^6/mL)	Day 2 Daily Growth	Relative Suspension Growth[a]	Relative Plating Efficiency[b]	Relative Total Growth[c]	Viable Counts	TFT Mutant Counts
Medium +S9	77 84	0.805	2.68	137 152	1.445	4.82	100.0	100.0	100.0	249 248 241	49 59 43
3-MCA 2.5 +S9	88 82	0.850	2.83	146 123	1.345	4.48	98.3	87.8	86.3	223 213 212	163 164 127
50 +S9	65 65	0.650	2.17	128 124	1.260	4.20	70.4	90.1	63.4	200 235 230	50 47 49
250 +S9	74 69	0.715	2.38	110 131	1.205	4.02	74.1	108.9	80.7	281 258 265	61 66 74
500 +S9	68 74	0.710	2.37	111 128	1.195	3.98	72.9	108.4	78.1	260 271 269	55 60 53
2500 +S9	82 76	0.790	2.63	110 108	1.090	3.63	74.0	98.6	73.0	231 254 243	71 69 68
5000 +S9	50 47	0.485	1.62	100 95	0.975	3.25	40.7	78.3	31.8	179 208 191	129 133 114

[a] Relative Suspension Growth = Cumulative cell count (CCC) in treatment group/ CCC in medium control.
[b] Relative Plating Efficiency = Mean number of VC colonies in treatment group/ Mean number of VC colonies in solvent control.
[c] Relative Total Growth = Day 2 Relative % Suspension Growth × Relative Plating Efficiency.

Appendix-B6. Sizing of TFT Mutants in Experiment A without S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Size Setting (mm)												
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3
Medium	67	39	37	30	16	7	1	0	0	0	0	0	0
	69	38	38	24	17	9	1	0	0	0	0	0	0
	74	55	48	25	19	8	2	0	0	0	0	0	0
EMS 250 nl/mL	257	213	188	144	75	15	6	4	1	2	0	0	0
	225	188	162	128	77	17	3	3	2	3	2	1	0
	237	207	191	134	78	14	5	8	4	2	3	1	0
50	70	42	47	36	26	14	3	0	0	0	0	0	0
	70	44	48	31	29	17	4	0	0	0	0	0	0
	74	46	49	34	26	17	4	0	0	0	0	0	0
250	64	39	34	27	19	15	6	0	1	0	0	0	0
	66	39	42	26	18	15	4	1	1	0	0	0	0
	65	37	40	23	20	15	4	1	0	0	0	0	0
500	89	59	58	45	33	19	5	1	1	1	0	0	0
	92	59	56	44	36	21	5	1	0	0	0	0	0
	89	58	59	45	34	17	5	0	0	0	0	0	0
2500	77	56	52	42	35	19	6	1	0	0	0	0	0
	84	43	51	36	28	17	7	0	0	0	0	0	0
	81	54	54	35	27	23	3	2	0	0	0	0	0
5000	99	74	65	56	39	19	7	0	0	0	0	0	0
	99	72	67	54	36	18	4	0	0	0	0	0	0
	98	68	71	45	38	21	2	2	0	0	0	0	0

Appendix-B7. Sizing of TFT Mutants in Experiment A with S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Size Setting (mm)												
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3
Medium	75	46	48	40	24	22	8	2	1	1	1	0	0
	86	63	58	43	24	14	1	1	1	0	0	0	0
	81	47	54	36	34	7	3	0	0	0	0	0	0
3-MCA 2.5	205	170	139	111	76	23	7	3	3	2	2	1	0
	208	168	143	113	69	23	5	2	2	0	0	0	0
	205	170	149	116	82	16	3	3	1	1	2	1	0
50	92	61	64	49	39	21	6	2	0	0	0	0	0
	80	54	57	43	40	22	5	0	0	0	0	0	0
	103	77	65	53	33	24	6	0	1	1	0	0	0
250	76	49	59	37	26	23	5	1	1	1	1	1	0
	94	68	67	51	30	24	8	2	1	1	1	0	0
	84	57	52	42	42	18	8	1	1	0	0	0	0
500	72	49	41	35	26	16	7	1	0	0	0	0	0
	68	37	41	28	22	17	6	1	0	0	0	0	0
	67	44	47	27	23	20	8	1	1	1	1	1	0
2500	89	59	58	40	30	21	7	1	0	0	0	0	0
	82	56	51	44	37	24	9	1	0	0	0	0	0
	91	64	59	41	32	29	6	1	0	0	0	0	0
5000	192	151	110	105	85	50	15	5	2	3	3	0	0
	221	171	120	91	61	30	10	3	2	1	1	0	0
	217	180	132	80	67	32	5	4	2	1	1	0	0

Appendix-B8. Sizing of TFT Mutants in Experiment B without S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Size Setting (mm)									
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Medium	61	43	33	29	24	11	3	0	0	0
	63	47	39	34	27	14	6	0	0	0
	66	41	37	28	22	14	1	0	0	0
EMS 250 nl/mL	229	194	186	165	108	25	2	1	0	0
	216	183	172	158	99	20	2	2	1	0
	214	204	186	144	91	26	2	1	1	0
50	66	48	39	35	22	19	9	0	0	0
	58	47	28	32	28	23	10	2	1	0
	64	40	40	26	31	22	12	3	0	0
250	55	30	28	24	11	8	7	2	0	0
	58	41	19	24	24	19	14	1	0	0
	49	40	30	14	23	20	6	1	0	0
500	59	50	43	38	31	23	2	0	0	0
	73	50	39	33	25	19	5	2	0	0
	70	44	41	34	33	12	11	0	0	0
2500	68	53	45	22	35	17	7	1	0	0
	59	40	32	30	29	18	8	4	0	0
	66	46	31	42	19	19	9	1	0	0
5000	109	79	63	53	54	42	6	1	0	0
	113	71	80	42	36	28	13	6	3	0
	96	87	52	62	42	21	30	1	0	0

Appendix-B9. Sizing of TFT Mutants in Experiment B with S9 Activation (Study #:1093-A06-2)

Treatment (μg/mL)	Size Setting (mm)									
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Medium	49	42	44	42	22	17	17	2	1	0
	59	33	26	36	33	34	19	3	1	0
	43	46	42	23	37	25	11	5	1	0
3-MCA 2.5	163	107	105	99	103	66	14	6	2	0
	164	143	129	121	91	71	23	2	2	0
	127	144	130	122	103	65	22	4	1	0
50	50	35	27	24	25	18	4	1	2	0
	47	34	24	29	24	19	13	3	3	0
	49	35	32	25	26	18	8	3	0	0
250	61	46	40	32	27	24	15	2	0	0
	66	58	48	31	39	20	11	4	0	0
	74	50	36	42	26	33	13	2	0	0
500	55	31	26	21	19	18	9	1	0	0
	60	40	33	23	24	13	3	1	0	0
	53	36	27	27	20	17	7	0	0	0
2500	71	52	39	33	35	28	14	2	0	0
	69	53	44	33	31	25	6	0	0	0
	68	48	42	39	32	28	13	1	0	0
5000	129	103	76	69	54	31	16	1	0	0
	133	85	65	50	45	22	8	1	1	0
	114	105	86	59	58	38	5	1	1	0

APPENDIX C. Raw Data of In Vivo Mouse Bone Marrow Micronuclei Test

APPENDIX C. Raw Data of In Vivo Mouse Bone Marrow Micronuclei Test

Genotoxicity of Ammonium Dinitramide (ADN) - Micronucleus Test
(Study Number: 1093-A06-3)

Animal #	Code #	Sex	Dose Group	PCE/NCE	X±SD	MN(%)	X±SD
1	15	M	Control	1.545		0.3	
2	19	M	Control	1.694		0.3	
3	21	M	Control	1.593		0.3	
4	55	M	Control	1.489		0.4	
5	1	M	Control	1.429	1.55±0.01	0.4	0.34±0.05
6	45	M	Cyclophosphamide (20 mg/kg)	0.359		2.3	
7	20	M	Cyclophosphamide (20 mg/kg)	0.417		2.0	
8	53	M	Cyclophosphamide (20 mg/kg)	0.304		2.4	
9	28	M	Cyclophosphamide (20 mg/kg)	0.484		2.8	
10	13	M	Cyclophosphamide (20 mg/kg)	0.323	0.38±0.07	3.2	2.54±0.47
11	58	M	ADN (750 mg/kg)	0.625		1.0	
12	40	M	ADN (750 mg/kg)	0.617		1.1	
13	41	M	ADN (750 mg/kg)	0.695		1.3	
14	52	M	ADN (750 mg/kg)	0.675		0.8	
15	16	M	ADN (750 mg/kg)	0.645	0.65±0.03	0.9	1.02±0.19
16	Dead	M	ADN (500 mg/kg)				
17	25	M	ADN (500 mg/kg)	1.056		0.9	
18	29	M	ADN (500 mg/kg)	1.074		1.0	
19	3	M	ADN (500 mg/kg)	0.872		0.6	

Animal #	Code #	Sex	Dose Group	PCE/NCE	X±SD	MN(%)	X±SD
20	23	M	ADN (500 mg/kg)	1.045	1.01±0.09	1.0	0.94±0.16
21	8	M	ADN (250 mg/kg)	1.147		0.8	
22	24	M	ADN (250 mg/kg)	1.124		0.7	
23	17	M	ADN (250 mg/kg)	1.348		0.7	
24	64	M	ADN (250 mg/kg)	1.104		0.6	
25	4	M	ADN (250 mg/kg)	1.201	1.18±0.10	0.7	0.70±0.07
26	2	M	ADN (125 mg/kg)	1.243		1.0	
27	44	M	ADN (125 mg/kg)	1.213		0.5	
28	62	M	ADN (125 mg/kg)	1.261		0.8	
29	26	M	ADN (125 mg/kg)	1.042		0.8	
30	38	M	ADN (125 mg/kg)	1.022	1.16±0.11	0.9	0.80±0.19
31	66	M	ADN (62.5 mg/kg)	1.359		0.5	
32	22	M	ADN (62.5 mg/kg)	1.435		0.4	
33	11	M	ADN (62.5 mg/kg)	1.770		0.4	
34	34	M	ADN (62.5 mg/kg)	1.648		0.4	
35	14	M	ADN (62.5 mg/kg)	1.506	1.54±0.17	0.4	0.42±0.04
36	18	F	Control	2.204		0.2	
37	36	F	Control	1.328		0.5	
38	37	F	Control	1.166		0.4	
39	42	F	Control	1.603		0.3	
40	63	F	Control	1.471	1.55±0.40	0.5	0.38±0.13
41	60	F	Cyclophosphamide (20 mg/kg)	0.565		2.5	

Animal #	Code #	Sex	Dose Group	PCE/NCE	X±SD	MN(%)	X±SD
42	39	F	Cyclophosphamide (20 mg/kg)	0.527		3.4	
43	47	F	Cyclophosphamide (20 mg/kg)	0.384		2.3	
44	49	F	Cyclophosphamide (20 mg/kg)	0.411		2.4	
45	46	F	Cyclophosphamide (20 mg/kg)	0.618	0.50±0.10	2.5	2.62±0.44
46	9	F	ADN (750 mg/kg)	0.634		0.9	
47	57	F	ADN (750 mg/kg)	0.579		1.3	
48	32	F	ADN (750 mg/kg)	0.696		0.8	
49	Dead	F	ADN (750 mg/kg)				
50	6	F	ADN (750 mg/kg)	0.630	0.63±0.05	1.0	1.0±0.22
51	Dead	F	ADN (500 mg/kg)				
52	27	F	ADN (500 mg/kg)	1.188		0.7	
53	43	F	ADN (500 mg/kg)	1.068		1.0	
54	50	F	ADN (500 mg/kg)	1.132		0.9	
55	5	F	ADN (500 mg/kg)	1.054	1.11±0.06	0.8	0.85±0.13
56	54	F	ADN (250 mg/kg)	1.336		0.6	
57	48	F	ADN (250 mg/kg)	1.281		0.6	
58	31	F	ADN (250 mg/kg)	1.140		0.5	
59	7	F	ADN (250 mg/kg)	1.186		0.7	
60	65	F	ADN (250 mg/kg)	1.380	1.26±0.10	0.6	0.60±0.07
61	35	F	ADN (125 mg/kg)	1.691		0.8	
62	10	F	ADN (125 mg/kg)	1.326		0.3	
63	61	F	ADN (125 mg/kg)	1.207		0.4	

Animal #	Code #	Sex	Dose Group	PCE/NCE	X±SD	MN(%)	X±SD
64	33	F	ADN (125 mg/kg)	1.423		0.5	
65	59	F	ADN (125 mg/kg)	1.560	1.44±0.19	0.4	0.48±0.19
66	Dead	F	ADN (62.5 mg/kg)				
67	30	F	ADN (62.5 mg/kg)	1.381		0.4	
68	12	F	ADN (62.5 mg/kg)	1.309		0.2	
69	56	F	ADN (62.5 mg/kg)	1.447		0.3	
70	51	F	ADN (62.5 mg/kg)	1.441	1.39±0.06	0.3	0.30±0.08

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PROTOCOLS FOR THE GENOTOXICITY ASSAYS OF AMMONIUM DINITRAMIDE (ADN)

Table of Contents:

I. Overall Objectives:

Research will be conducted to determine the potential genotoxicity associated with the exposure to ammonium dinitramide (ADN), an explosive chemical that is being considered for potential military and space application.

Three short-term genotoxic assays will be used to examine the genotoxicity of ammonium dinitramide, which include:

1. Salmonella/Mammalian microsome reverse mutation assay
   - per EPA (TSCA) Health Effects Testing Guidelines
   (40 CFR 798.5265)

2. Mouse lymphoma assay
   - per EPA (TSCA) Health Effects Testing Guidelines
   (40 CFR 798.5300)

3. *In vivo* mouse bone marrow micronucleus test
   - per EPA (TSCA) Health Effects Testing Guidelines
   (40 CFR 798.5395)

II. Protocol for the Genotoxicity Assay of Ammonium Dinitramide — Salmonella/Mammalian Microsome Mutagenesis Assay

Study Title: Genotoxicity Testing of Ammonium Dinitramide (ADN) Using the Salmonella/Mammalian Microsome Mutagenesis Assay

Sponsor's Rep:
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Program Manager, THRU

Contract Laboratory:
Cell. & Mol. Toxicology Program
ManTech Environmental Technology, Inc.
2 Triangle Dr.
Research Triangle Park, NC, 27709

Proposed Schedule:

1. Starting Date: March 15, 1994
2. Completion Date: June 15, 1994
3. Final Report Date: July 15, 1994

Approvals:

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Dr. Darol Dodd
Sponsor's Rep.
(THRU)

Mike Ray — Date 3/8/94
Mike Ray
QA Manager

Sheela Sharma — Date 3/8/94
Dr. Sheela Sharma
Study Director

II.A. Purpose:

Testing will be conducted to determine the potential genotoxicity associated with the exposure to ammonium dinitramide (ADN), using The Salmonella/Mammalian microsome reverse mutation system which measures the reversion from his- (histidine dependent) to his+ (histidine independent) reversion induced by chemicals which cause base changes or frameshift mutations in the genome of this organism. This assay will be used to detect the mutations induced by the test agent and performed in accordance with the EPA/TSCA Health Effects Testing Guidelines 40 CFR 798.5265.

II.B. Background

A reverse mutation assay using Salmonella typhimurium detects mutations in a gene of a histidine requiring strains to produce a histidine independent strain of this organism. A reverse mutation can be achieved by base pair changes, which may occur at the site of the original mutation or at a second site in the chromosome; or by frameshift mutations resulting from the addition or deletion of single or multiple base pairs in the DNA molecule.

In this assay, bacteria are exposed to the test agent with and without a metabolic activation system and plated onto minimum agar medium which is deficient in histidine. After a suitable period of incubation, revertant colonies are counted and compared with the number of spontaneous revertants in an untreated and/or vehicle control culture. The mutagenicity of the test agents is evident by the increased number of revertants.

II.C. Justification of the Test

Salmonella/mammalian microsome mutagenesis is a microbial reverse mutation assay widely used in genetic toxicology studies. The system uses genetic variants (histidine independent) that can be easily detected among the large population of nonmutated cells (histidine dependent). The incorporation of activation systems, such as mammalian microsome greatly improves the efficacy of the assay. The high sensitivity and specificity of this assay in the detection and verification of mutagens/carcinogens have been well established, and it is the representative microbial mutagenesis test in a battery of short term genotoxicity assays. A significant percentage of chemicals that show a mutagenic response are potential animal and human mutagens and carcinogens (Tennant, et.al 1987)

II.D. Test Methods

1. Tester strains:

Four tester strains will be used in this assay, which include TA1535 and TA100 for the detection of base pair mutagens, and TA1537 and TA98 for the detection of frameshift mutagens. The tester strains are from Dr. Bruce N. Ames at University of California, CA.

2. Confirmation of the genotypes of the tester strains:

The following genotypes will be confirmed in each tester strain based on the methods described by Maron and Ames (1983) prior to the mutagenesis study:

a. Requirement of histidine for growth (His-)
b. Sensitivity to Crystal violet (rfa mutation)
c. Sensitivity to U.V. light (uvrB mutation)
d. Resistance to ampicillin (R factor)
e. Spontaneous revertant

3. Bacteria growth:

Fresh culture of the tester strains will be used for each assay.
The bacteria are cultured in nutrient broth at 37°C water bath with shaking for 10-12 hours to reach the late exponential or early stationary phase of growth (10^8-10^9 cells/ml).

4. Metabolic activation:

The test compound will be examined both in the presence and absence of an appropriate metabolic activation system. The most commonly used activation system in this assay is S9 mixture, a cofactor supplemented postmitochondrial fraction prepared from the liver of rats treated with enzyme inducers such as Aroclor-1254.

Male Sprague-Dawley rats (b.w. ~200 g) are treated with Aroclor 1254 by i.p. injection at a dose of 200 mg/kg body weight. Five days later animals are sacrificed by cervical dislocation and the livers are collected, and homogenized in 0.15 M KCl. The homogenate is centrifuged at 9000 g for 10 minutes. The supernatant is aliquoted and stored at -80°C and used as the S9.

5. Test agent:

Ammonium dinitramide will be freshly dissolved in sterile distilled water to the required concentrations. A preliminary range finding test including 5 log doses (with 5 mg/plate as the top dose) will be conducted in TA100 for the dose selection. Toxicity will be assessed by the reduction in the spontaneous revertants per plate, and /or a clearing of the background lawn. Five concentrations with adequate intervals will be selected and tested in the mutagenesis.

6. Controls:

In each assay, following concurrent controls will be set up:

a. Negative and solvent controls:

Untreated cultures with and without S9 mixture are set up as negative control. They are used for the measurement of spontaneous revertants, which will serve as the background level of reverse mutation. DMSO controls will also be included in each assay.

b. Positive control:

Positive controls with known mutagens shall ensure the responsiveness of the tester strains as well as the efficacy of the activation system. Sodium azide (CAS 26628-22-8)(without S9) will be the positive control for TA1535 and TA100. The positive controls for TA98 and TA1537 are 2-aminofluorene (CAS 153-78-6)(with S9) and 9-aminoacridine (CAS 90-45-9)(without S9), respectively. The above positive control agents will be dissolved in DMSO.

7. Mutagenesis assay (plate incorporation method):

All dose levels (with and without S9 mixture) will be set up in triplicates. 0.1 ml of the culture is added to 2 ml of top agar which is melted and held in a 45°C heating block, along with 0.1 ml of the test agent, and 0.5 ml of S9 mixture (in S9+ plates only). The contents are mixed and then poured onto the surface of a minimum glucose agar plate and spread out evenly. The top agar is allowed to solidify and the plates are inverted and incubated at 37°C for 48 hours. The number of revertants per dish is counted by an automatic colony counter.

II.E. Data collection and reporting

The experimental data will be entered into a predesigned Lotus 1-2-3 spreadsheet and analyzed. The following specific information will be reported for the Salmonella mutagenesis assay: 1. Tester strains used (results of genotypic confirmation), 2. Metabolic activation system used (source, amount, cofactors, method for preparation), 3. Dose levels and the rationale for their selection, 4. Positive and negative controls, 5. Individual plate counts, means, and standard deviation, and 6. Dose response relationship if applicable.

II.F. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous revertant frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. A sufficient number of nontoxic concentrations have been tested.

c. The strain-specific positive mutagens significantly increase the revertant in the corresponding strains.

2. Criteria for interpretation:

a. Positive result:

A compound will be considered positive in this assay if a dose-dependent increase in the number of revertants is observed in three concentrations, and the highest increase in TA1535 and TA1537 is equal to three times the spontaneous control value or the highest increase in TA98 and TA100 is equal to two times the spontaneous level (Brusick, 1989). Sometimes the precise fold increase will not be necessary if a clear dose-dependent pattern is noted over several concentrations.

A positive result in Salmonella/microsome mutagenesis indicates that under the experimental conditions, the test compound induces point mutation by base changes or frameshift in the genome of this organism.

b. Negative result:

A test agent will be considered negative in this assay if the criteria for positive response are not met, and the tester strains are sensitive to the positive mutagens.

A negative result indicates that under the experimental conditions, the test compound is not mutagenic in Salmonella typhimurium.

III. Protocol for the Genotoxicity Assay of Ammonium Dinitramide — Mouse Lymphoma Cell Mutagenesis Assay

Study Title: Genotoxicity Testing of Ammonium Dinitramide (ADN) Using the Mouse Lyphoma Cell Mutagenesis Assay

Sponsor's Rep:
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Contract Laboratory:
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Research Triangle Park, NC, 27709

Proposed Schedule:

1. Starting Date: March 15, 1994
2. Completion Date: June 15, 1994
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Approvals:

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Mike Ray — Date 3/8/94
Mike Ray
QA Manager

Sheela — Date 3/8/94
Dr. Sheela Sharma
Study Director

III.A. Purpose:

Research will be conducted to determine the potential genotoxicity associated with the exposure to ammonium dinitramide (ADN), using a Mammalian cell culture system to detect mutations. The L5178Y mouse lymphoma-TK assay detects the mutations at the thymidine kinase locus and will be used to test the mutagenicity of the test agent in mammalian cell cultures. The assay will be performed in accordance with the EPA/TSCA Health Effects Testing Guidelines 40 CFR 798.5300.

III.B. Background

Thymidine monophosphate (TMP) occupies a unique position in DNA replication. Of the four principal deoxyribonucleotide monophosphates, TMP alone does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition, which serves as a regulator for DNA synthesis. If the TMP is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the "salvage" enzyme thymidine kinase (TK), which normally phosphorylates thymidine to TMP in most mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues. In the mouse lymphoma cell forward mutation assay, the TK-competent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After a certain period of expression, the cells are shifted to a selective medium containing the lethal analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). Only the mutant cells (TK-/-) can survive under the selection condition, and the mutagenicity of the test compound is evident by the increase in the number of mutants.

III.C. Justification For the Test System

L5178Y mouse lymphoma cell mutagenesis system is one of the most commonly used mammalian cell mutagenesis assays in genetic toxicology study. Like other mutagenesis tests, this system uses genetic variants (TK-) which are easily detected among a large population of nonmutated cells (TK +/-). Compared with other mammalian cell mutagenesis systems such as the CHO-hgprt assay, the mouse lymphoma cell assay has the following advantages: 1) the assay is able to detect not only the gene mutations involving base substitution, frameshifts, small deletion and rearrangement within the gene, but also the chromosomal (multiple gene) mutations and multilocus deletions which are less detectable in the CHO-hgprt system. 2) the doubling time of the cells is shorter (10-11 hours), 3) the expression time is shorter (2 days), and 4) the suspension culture is easier to handle.

III.D. Test Methods

1. Cells and culture maintenance:

The L5178Y $TK^{+/-}$ mouse lymphoma cells, clone 3.7.2C, obtained from Dr. Donald Clive of Burroughs Wellcome Co. (Research Triangle park, NC) will be used. The cells used in the mutagenesis assay should have a high cloning efficiency and low spontaneous mutation frequency. The cells will be maintained as suspension culture in Fischer's (F_{10p}) media (GIBCO) in culture flasks equilibrated with 5% CO_2/95% air and incubated at 37°C in a rotary shaker.

Each week the cells will be grown in the F_{10p} media containing THMG (thymidine, hypoxanthine, methotrexate and glycine) to select against newly arising TK-/- mutants, and then placed in the F_{10p} media containing THG (thymidine, hypoxanthine, and glycine) for 1-3 days prior to use in mutagenesis study.

2. Metabolic activation system:

Cells will be exposed to the test agent both in the presence and absence of an appropriate metabolic activation system. Cofactor- supplemented liver S9 from Aroclor-induced rats will be prepared as described by Mitchell et al (1988) and used in each assay.

3. Test agent:

Ammonium dinitramide will be freshly dissolved in distilled water prior to each use. A preliminary range finding experiment will be conducted using 10 doses over a 3-4 log range with 5000 ug/ml as the top concentration. The procedures for range finding are identical to that used for mutagenesis except that the cultures are terminated after 24-48 hours without further cloning. The toxicity is indicated by the decrease of cell number in the suspension culture compared with that in untreated control. Four to five concentrations will be selected based on the range finding data and used in the mutagenesis assay. The highest dose should produce a low level of survival (approximately 10%), and the survival in the lowest dose should be the same as the negative control.

4. Controls:

Negative control without treatment and positive control with known mutagens should be included in each assay. Ethyl methanesulfonate (EMS, CAS 62-50-0, without S9 mixture) and 3-methylcholanthrene (3-MCA, CAS 56-49-5, with S9 mixture) will be used as the positive controls. Both mutagens are dissolved in DMSO, and corresponding solvent control will also be included.

5. Mutagenesis assay:

a. Exposure:

Cells ($6x10^6$ cells in 10 ml medium for each culture) are treated with test agents with and without S9 mixture, and incubated at 37°C with rotation for 4 hours. Chemicals are removed and cells are washed twice by centrifugation then resuspended in non-selective medium at a density of $3x10^5$ cells/ml, and maintained in roller drum for 2 days at 37°C.

b. Expression:

The 2 day maintenance after exposure is the expression period for mutation. During this period, cell density is checked daily and adjusted to $3x10^5$ cells/ml.

c. Cloning:

On the second day of expression, cells are seeded onto soft agar medium to determine the survival and the mutation frequency. For each dose group, 3 cultures containing 200 cells/dish in non-selective medium are set up for viability measurement, another set of 3 cultures with $1x10^6$ cells/dish in selective medium containing TFT are used for mutant counting. Dishes are incubated at 37°C in an atmosphere of 5% CO_2/95% air.

d. Colony counting:

Colonies are counted 11-12 days after cloning using an automatic colony counter. The mutant frequency is calculated and adjusted based on the survival percentage.

III.E. Data collection and reporting

All the original records about cell maintenance, medium and chemical preparation, cell counts, S9 preparation, details of experimental set-up of range finding and mutagenesis assay will be kept in standard forms. Results will be expressed in tabular form which include colony forming efficiency (CFE %), relative CFE (RCFE), number of mutants, mutation frequency (MF) and relative mutation frequency (RMF) for each culture. Specifically for the mouse lymphoma cell mutagenesis assay, the following information will be included in the report: 1) cells (type, number of cultures, methods for maintenance), 2) test agents (dose selection and rationale), and 3) experimental conditions (incubation temperature, CO_2 concentration, treatment schedule, cell density, metabolic activation system and its preparation, positive and negative controls, length of expression, selective agent and concentration).

III.F. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous mutation frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. The test system is sensitive to the known mutagen as judged by the results in the concurrent positive control cultures.

2. Criteria for interpretation:

a. Positive result:

A test agent will be considered positive, if it induces a statistically significant dose-related increase in the mutant frequency, or generates a reproducible and statistically significant increase in the mutant frequency for at least one concentration.

A positive result in mouse lymphoma cell mutagenesis assay indicates that under the experimental

conditions, the test compound induces gene mutation in the cells used.

b. Negative result:

A test agent which does not produce either a statistically significant dose-related increase, or a reproducible and statistically significant increase of the mutant frequency in any one of the concentrations tested will be considered nonmutagenic in this system.

A negative result indicates that under the experimental conditions, the test compound does not produce gene mutation in the cells used.

3. Statistical analysis:

The toxicity of the test agent will be indicated by a decrease in CFE (or relative CFE, RCFE), which will be determined as follows:

CFE (%) = number of colonies/number of cells plated x 100%
RCFE = CFE in treated culture/CFE in negative control

The mutagenicity of the test agent will be evident from the increase in mutation frequency (MF, or expressed as relative mutation frequency, RMF) based on the number of mutants and adjusted by the survival fraction of cells:

MF= No. of mutants/ No. of clonable cells x 10^6
RMF = MF in treated culture/MF in negative control

The differences in CFE and MF between control and treated cultures are evaluated by a two-tail Student's t-test. The dose-dependent response is examined by a linear regression.

IV. Protocol for the Genotoxicity Assay of Ammonium Dinitramide
— In Vivo Mouse Bone Marrow Micronucleus Test

Study Title: Genotoxicity Testing of Ammonium Dinitramide (ADN) Using the *In Vivo* Mouse Bone Marrow Micronucleus Test

Sponsor's Rep:
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Contract Laboratory:
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Proposed Schedule:

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Dr. Sheela Sharma
Study Director

IV.A. Objectives:

Research will be conducted to determine the potential genotoxicity associated with the exposure to ammonium dinitramide (ADN), an explosive chemical that is being considered for potential military and space application. The *in vivo* mammalian micronucleus test, which detects the damage of chromosome or mitotic apparatus caused by chemicals, will be used to examine the chromosome-damaging effect of the test agent. Polychromatic erythrocytes (PCE) in bone marrow of rodents are used in this assay. The assay is based on an increase in the frequency of micronucleated PCEs in bone marrow of the treated animals. The assay will be conducted according to the EPA/TSCA Health Effects Testing Guidelines 40 CFR 798.5395.

IV.B. Background

Micronuclei are small particles consisting of acentric fragments of chromosome or entire chromosomes, which lag behind at anaphase of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. The clastogenic effect or mitotic apparatus damaging effect of the test agent will be evident by the increased frequency of micronucleated PCEs in the bone marrow.

IV.C. Justification of the Test

The *in vivo* micronucleus test in the mice bone marrow polychromatic erythrocytes is a routine genetic toxicology technique for chromosome damage. The test agent is metabolized *in vivo* in the experimental animals. The assay is technically simpler than the traditional metaphase analysis of chromosome aberration. Using the polychromatic erythrocyte, which lack the main nuclei, greatly facilitates the visualization of the micronuclei and improves the accuracy of the assay. The predictive value of the assay in carcinogen identification has been well documented (Arlett, et.al. 1989; Heddle, et al. 1983; Schmid, 1976).

IV.D. Test Methods

1. Experimental animals:

Swiss CD-1 mice, both sex, 8-10 week old will be used in the study. Animals will be procured from Charles River Laboratory. Five males and five females will be included for each test group. Animals are quarantined for 1 week, and then randomized and assigned to treatment and control groups.

2. Test agent:

Ammonium dinitramide will be dissolved in distilled water prior to each use and administered by single intraperitoneal (i.p.) injection. In the initial assessment of cytotoxicity, two doses of 1000 and 5000 mg/kg will be used. The cytotoxicity will be judged by a decrease in the ratio of PCEs/NCEs (normochromatic erythrocytes) in the bone marrow. Three doses will be used in the dose-response study.

3. Controls:

Concurrent negative control (without treatment) and positive control (cyclophosphamide, CAS 6055-19-2, a known micronucleus inducer dissolved in physiological saline) will be included. Animals in the negative control group are used for the measurement of background frequency of micronucleated cells, and the positive control is used to verify the responsiveness of the test system. Saline controls are also included.

4. Dosing and sampling:

Based on the cytotoxicity assessment data (THRU) of the test agent, the experiment will be conducted by either a one-dosing, one-sampling or a multiple dosing one-sampling protocol. Briefly, the test compounds will be dissolved in distilled water and administered by either a single or multiple intraperitoneal (i.p.) injection. Twenty-four hours after the injection, mice are sacrificed and bone marrow cells are collected.

5. Preparation of bone marrow smears:

The bone marrow cells will be collected and suspended in 3 ml fetal bovine serum (FBS). After centrifugation at 1000 rpm for 5 minutes, the pellet will be resuspended with a few drops of (FBS), and smears prepared on standard microscopic slides.

6. Staining of the slides:

The slides will be stained the next day of preparation by May-Gruenwald and Giemsa solution as described by Schmid et al (1975). The slides will be treated with xylene for 5 minutes and then embedded with coverslips.

7. Micronuclei observation:

The frequency of micronucleated cells will be observed in 1000 polychromatic erythrocytes (PCE) per animal. The PCEs/NCEs ratio is determined by counting 1000 erythrocytes, and used as the indicator of toxicity. Micronuclei are some round bodies in cytoplasm with a diameter of 1/20 to 1/5 of an erythrocyte. They stain intensively, similar to the staining of the main nuclei in the nucleated cells.

IV.E. Data collection and reporting

All the original observation for micronucleated cell frequency and PCEs/NCEs ratio will be recorded in standard scoring forms including the criteria for scoring of micronuclei. Individual data will be presented in a tabular form which includes positive control, negative control, solvent control and treatment groups. The number of PCEs scored, the number of micronucleated PCEs, the percentage of micronucleated PCEs, and the ratio between PCEs and NCEs will be listed separately.

The test report for micronucleus assay will also include the following specific information: 1) experimental animals (species, age, body weight, sex, number), 2) test agent (vehicle, doses and rationale

for dose selection), 3) treatment and sampling schedule, 4) toxicity data, 5) positive and negative controls, 6) procedures for slide preparation and staining, and 7) criteria for micronuclei identification.

IV.F. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The background frequency of micronucleated cells is in the normal range as reported in the literature or within the laboratory's historical range.

b. The test system is sensitive to the known mutagen as judged by the results in the concurrent positive control animals.

2. Criteria for interpretation:

a. Positive result:

The test agent will considered positive in this assay, if it induces a statistically significant dose-related increase in the number of micronucleated PCEs, or a reproducible and statistically significant increase in the micronucleated PCE frequency for at least one concentration.

A positive result in micronucleus test indicates that under the experimental conditions, the test compound induces micronuclei by damage of either chromosome or mitotic apparatus.

b. Negative result:

A test agent which does not produce either a statistically significant dose-related increase in the number of micronucleated PCEs, or a reproducible and statistically significant increase in the micronucleated PCE frequency in any one of the concentrations tested will be considered nonmutagenic in this system.

A negative result indicates that under the experimental conditions, the test compound does not produce micronuclei in the bone marrow of the test species.

3. Statistical analysis:

The differences in the micronucleated PCE frequency and the ratio of PCEs/NCEs among treated and control animals are statistically evaluated by Chi-square analysis, and the dose-dependent response is examined by linear regression.

V. Identification, Handling and Storage and of the Test Agents

The test agent, ADN will be provided by the THRU. A total of 10 *g* is required for completing the three assays including any confirmatory tests. The identification, stability, purity, chemical and physical properties of the material will be the responsibility of THRU. The compound is highly soluble in water (500 g/liter) and stable below 50°C. It will be stored at the Hazard Materials Laboratory (HML) in sealed dark or opaque glass container and at room temperature, avoiding direct sunlight or sudden temperature rise. The primary routes of exposure include skin absorption, ingestion and inhalation. Safety glasses, rubber gloves, and protective clothing will be used during handling.

VI. Good Laboratory Practice and Quality Assurance

All assays will be conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA/TSCA) Good Laboratory Practice (GLP) Standards as defined in the Federal Register (40 CFR, Part 792, 1992) and the TSCA Test Guidelines (40 CFR 798.5265, 40 CFR 798.5300, and 40 CFR 5395, 1992). All the procedures are performed in accordance with the Standard Operating Procedures (SOPs) of ManTech Environmental for the Salmonella/microsome mutagenesis assay, L5178Y mouse lymphoma cell mutagenesis and mouse bone marrow cell micronucleus test.

The Quality Assurance Officer, Mr. Mike Ray will document inspections on all procedures used in this study. After the initiation of the study, any modifications of the protocol will be in the form of Protocol Amendments, which will state the specific modifications and the reasons for the modifications.

VII. Schedule

In the starting phase (about 4 weeks), all the test agents, media, equipment, cells, tester bacteria and animals will be ordered. The genotypes of the tester strains will be confirmed, including the maintenance of cells at no cost to THRU. The range finding studies for dose selection will also be conducted in this period. All three genotoxicity assays will be completed within 3 months (12 weeks). Independent confirmatory experiments will be conducted for the mouse lymphoma cell mutagenesis assay and Salmonella/microsome mutagenesis assay depending upon the option of THRU. Another month will be contributed to data analysis and report preparation.

VIII. Reports

Brief monthly technical progress reports will be prepared and submitted to Dr. Darol Dodd, program director, THRU, no later than the 12th of each month, indicating the stage of completion of the requested genotoxic assays. The study will be initiated on March 15, 1994, and the dates for monthly reports will be April 12, May 12, and June 12, 1994. The final report will be submitted by July 15, 1994.

IX. Deliverables

In addition to the slides and raw data from each of the assays, duplicate final reports presenting the pertinent findings of all three genotoxic assays will be prepared according to the format designated by the THRU. An additional report formatted in a manuscript form suitable for publication in "Environmental Molecular Mutagenesis Journal" will also be provided. Along with the completed reports and the appended study data a DOS compatible disk copy of all reports shall be provided. The reports and all supporting documents will be delivered to Dr. Darol Dodd at the THRU.

X. Key Personnel

Principal Investigator: Songyun Zhu, Technical Scientists: Elisabeth Korytynski, Lashawn Poinsette, and Merrie Burnett.

XI. References

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